

The grey zone between type 1 and type 2 diabetes

- genetic aspects of diabetes in adults

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To my family

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PUBLICATIONS

This thesis is based on the following publications and additional unpublished data.

- Study I** Andersen MK, Lundgren V, Turunen JA, Forsblom C, Isomaa B, Groop PH, Groop L, Tuomi T. Latent autoimmune diabetes in adults differs genetically from classical type 1 diabetes diagnosed after the age of 35 years. *Diabetes Care*. 2010;33(9):2062-4.
- Study II** Andersen MK, Lundgren V, Isomaa B, Groop L, Tuomi T. Association of variants in *HLA-DQA1-DQB1*, *PTPN22*, *INS*, and *CTLA4* with GAD autoantibodies and insulin secretion in nondiabetic adults of the Botnia Prospective Study. *European Journal of Endocrinology*. 2012;167(1): 27-33.
- Study III** Andersen MK, Härkönen T, Forsblom C, Groop PH, Knip M, Tuomi T. Zinc transporter type 8 autoantibodies (ZnT8A): prevalence and phenotypic associations in latent autoimmune diabetes in adults and type 1 diabetes diagnosed >35 years. *Autoimmunity*, 2012 in press.

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ABBREVIATIONS

AUC	Area under the curve
BMI	Body-mass index
CI	Confidence interval
CIR	Corrected insulin response
CTLA4	Cytotoxic T-lymphocyte-associated antigen
CV	Coefficient of variation
DASP	Diabetes Autoantibody Standardization Program
FCRL3	FC receptor-like protein 3
FH _{T1}	Family history of type 1 diabetes
FPG	Fasting plasma glucose
fS	Fasting serum
FTO	Fat- and obesity-associated
GADA	Glutamic acid decarboxylase autoantibodies
GLIS3	GLI-similar 3
GWAS	Genomewide association studies
HbA _{1c}	Haemoglobin A1c
HDL	High density lipoprotein
HHEX	Haematopoietically expressed homeobox
HLA	Human leukocyte antigen
HR	Hazard ratio
IA-2A	Islet antigen 2 autoantibodies
IAA	Insulin autoantibodies
ICA	Islet cell antibodies
IFIH1	Inteferon induced with helicase C domain 1
IL27	Interleukin 27
IL2RA	Interleukin 2 receptor alpha
INS	Insulin gene
IU	International units
KCNQ1	Potassium voltage-gated channel, subfamily Q, member 1
LADA	Latent autoimmune diabetes in adults
LADA _{high}	LADA with GADA levels within the highest quartile
LADA _{low}	LADA with GADA levels within the lowest quartile
LADA _{mid}	LADA with GADA levels within the middle quartiles
LD	Linkage disequilibrium
LDL	Low density lipoprotein
LYP	Lymphoid tyrosine phosphatase
mRNA	Messenger ribonucleic acid
OGTT	Oral glucose tolerance test
OR	Odds ratio
PCR	Polymerase chain reaction
PPARG	Peroxisome proliferator-activated receptor γ
PTPN22	Protein tyrosine phosphatase, non-receptor type 22
RAF	Risk allele frequency
RU	Relative units

Abbreviations

SD	Standard deviation
SEM	Standard error of the mean
SH2B3	SH2B adaptor protein 3
SLC30A8	Solute carrier family 30, member 8
SNP	Single nucleotide polymorphism
T1D	Type 1 diabetes
T1D _{<20}	Type 1 diabetes diagnosed before the age of 20 years
T1D _{>35}	Type 1 diabetes diagnosed after the age of 35 years
T2D	Type 2 diabetes
TCF7L2	Transcription factor 7, like 2
THADA	Thyroid adenoma-associated
TSPAN8	Tetraspanin 8
WHO	World health organisation
VNTR	Variable number of tandem repeats
ZnT8	Zinc transporter type 8
ZnT8A	Zinc transporter type 8 autoantibodies
ZnT8A _R	ZnT8A subtype reacting with the arginine epitope
ZnT8A _{RW}	ZnT8A subtype reacting with both the arginine and the tryptophan epitope
ZnT8A _W	ZnT8A subtype reacting with the tryptophan epitope

ABSTRACT

The incidence and prevalence of diabetes is increasing worldwide at alarming rates, making the disease a major health burden. Enhanced understanding of the disease aetiology is therefore extremely important as it would improve the treatment, and possibly the prediction and prevention of diabetes. Diabetes is a complex disease with two major subtypes, the autoimmune type 1 (T1D) and the metabolic type 2 diabetes (T2D). This diagnostic subdivision is, however, especially in adults, intricate, and clinical evidence even suggests an overlap between T1D and T2D. Autoantibodies are a hallmark of T1D but are also detectable in 3 to 11% of European patients diagnosed with T2D. This autoantibody-positive diabetic subtype is termed latent autoimmune diabetes in adults (LADA) and is considered by the WHO a slowly progressive form of T1D. This classification of LADA is, however, based on comparison with T1D diagnosed in childhood, whereas studies comparing LADA and T1D patients diagnosed in a similar age range are lacking. A clear genetic predisposition exists for both T1D diagnosed <15 years and T2D, and to date several candidate genes have been identified for both subtypes, contributing to increased knowledge of the biological mechanisms underlying diabetes. With respect to LADA and adult-onset T1D, much less is known about genetic predisposition.

The aims of the study were 1) to compare LADA and adult-onset T1D patients with respect to clinical characteristics, prevalence and phenotypic associations of zinc transporter type 8 autoantibodies (ZnT8A), and associations with selected gene variants predisposing to T1D or T2D, 2) to investigate genetic heterogeneity in LADA related to glutamic acid decarboxylase autoantibody (GADA) reactivity level and family history of T1D, and 3) to assess the phenotypic associations of the T1D-susceptibility gene variants in LADA patients and in initially non-diabetic adults with emphasis on insulin secretion, prevalence of GADA, and progression to non-insulin dependent diabetes.

Patients with T2D, LADA, and T1D diagnosed at >35 or at <20 years as well as non-diabetic subjects were recruited as part of the Botnia Study or the FinnDiane Study. Diabetes was diagnosed based on WHO criteria. Fasting C-peptide concentration <0.2 nmol/l at the time of the investigation and initiation of insulin treatment within 6 months after diagnosis were included as criteria for diagnosis of T1D. LADA was defined as initial diagnosis of T2D, >35 years at diagnosis, GADA positive, and no insulin treatment in the first 6 months after diagnosis. Genetic susceptibility to T1D was represented by variants in *HLA-DQB1*, *INS* (rs689), *PTPN22* (rs2476601), and *CTLA4* (rs3087243), and genetic susceptibility to T2D was represented by variants in *TCF7L2* (rs7903146), *SLC30A8* (rs13266634), *KCNQ1* (rs2237895), *PPARG* (rs1801282), and *FTO* (rs9939609).

Variants in *HLA-DQB1*, *PTPN22*, and *TCF7L2* were associated with LADA in general, and variants in *CTLA4* and *SLC30A8* were associated with LADA with high and with low GADA levels, respectively. Regarding the frequency of both T1D- and T2D-susceptibility variants, LADA differed from adult-onset T1D patients. We also observed, immunologically, differences between LADA and adult-onset T1D patients. Hence, the ZnT8A were significantly more prevalent among LADA patients but associated with shorter disease duration only in the adult-onset T1D patients. Among the LADA patients, increased GADA reactivity was associated with higher frequencies of the T1D-susceptibility gene variants (*HLA-DQB1*, *PTPN22*, and *CTLA4*), lower frequency of the T2D-susceptibility gene variants (*SLC30A8* and *FTO*), lower insulin secretion, and lower BMI, whereas family history of T1D was associated with increased frequencies of *HLA-DQB1*-risk genotypes and reduced frequencies of *TCF7L2*-risk genotypes.

In non-diabetic adults followed prospectively, HLA-DQ and *PTPN22* risk genotypes were associated with the presence of GADA. These associations were accentuated in subjects with a family history of T1D, and in this group even the *INS*-risk genotype was associated with GADA. Furthermore, non-diabetic adults with a higher number of T1D-susceptibility genotypes (*HLA-DQ*, *PTPN22*, *INS*, and *CTLA4*) were unable to increase the insulin secretion to a level similar to those with a lower number of risk genotypes in response to the reduced insulin sensitivity at follow-up. However, the T1D-susceptibility genotypes were not associated with progression to non-insulin-dependent diabetes in these initially non-diabetic adults. In LADA patients, both an increased number of T1D-susceptibility genotypes and the T2D-associated *SLC30A8* R325W variant were associated with reduced insulin secretion. With respect to the T1D-susceptibility genotypes, the same tendency was observed among T2D patients.

In conclusion, LADA shared a genetic predisposition with both T1D and T2D. And both family history of T1D and level of GADA reactivity contributed to heterogeneity in LADA. Comparison of LADA and adult-onset T1D patients revealed genetic, phenotypic, and immunological differences. Our data suggest that LADA, rather than an age-related extension of T1D, is a slowly progressive form of autoimmune diabetes distinct from T1D. Furthermore, T1D-susceptibility gene variants signify a mild beta-cell destruction in non-diabetic adults as well as in LADA and T2D patients.

REVIEW OF THE LITERATURE

The prevalence of diabetes has reached epidemic proportions, making this disease a huge health burden on society in both developed and developing countries [1]. Worldwide, 285 million people were estimated to have diabetes in 2010, corresponding to a prevalence of 6.4%. By 2030, it is estimated that the number affected will rise to 439 million, equal to 7.7% of the world's adult population [2]. Its extreme increase in prevalence and incidence over the last decades is explained primarily by changes in lifestyle and environmental exposures.

Diabetes results from disruption of the glucose homeostasis. The disease can cause micro-vascular complications such as retinopathy, nephropathy, and neuropathy, macro-vascular complications such as coronary heart disease, cerebrovascular disease, and peripheral vascular disease, and ultimately death if left untreated. On the molecular level, the specific cause of diabetes is poorly understood. Improved understanding of the aetiology of diabetes is of great importance, as it would improve treatment and prevention possibilities.

1. DIABETIC SUBTYPES

Diabetes is a complex and heterogeneous disease with a number of subtypes. These include T1D, T2D, LADA, maturity onset diabetes of the young, gestational diabetes, neonatal diabetes, and maternally inherited diabetes and deafness [3-6]. The biological mechanisms underlying the development of diabetes include reduced insulin secretion, and increased insulin resistance in skeletal muscles, adipose tissue, and in the liver. These two mechanisms contribute to a varying degree to the pathogenesis of the different diabetic subtypes.

1.1. Type 1 diabetes

Among all cases of diabetes in Finland, T1D accounts for approximately 15%. The disease incidence varies about 100-fold across the world. Finland has the highest incidence, with >50 per 100 000 in <15-year-olds, whereas the rate in Venezuela and China is only 0.1 to 4.5 per 100 000 [7, 8]. This geographical incidence variability is thought to be a result of differing genetic backgrounds and environmental exposures. The changing environment also may be the underlying reason for the increasing incidence rate observed over the last three decades worldwide [7-9]. This is illustrated by the reduced frequency of newly diagnosed children with high-risk HLA genotypes, despite an unchanged frequency of these genotypes in the general population, indicating that the environmental pressure has increased and predisposes less genetically susceptible individuals to disease [8, 10].

In most cases, T1D is caused by autoimmune destruction of the insulin-producing beta-cells in the pancreas, which is mediated by infiltrating T-cells. Figure 1 depicts the most

recognized model of T1D development. This process is initiated by the interaction between susceptibility genes and environmental predisposing factors, and this triggers the autoimmune reaction. The first molecular markers of this process are autoantibodies directed towards insulin (IAA), followed by autoantibodies towards islet cells (ICA), glutamic acid decarboxylase (GADA), tyrosine phosphatase-like IA-2 protein (IA-2A), and zinc-transporter type 8 (ZnT8A) [11-13]. These autoantibodies are apparent in the early stages of the disease process. In this process, destruction of the beta-cells initially leads to loss of the first-phase insulin response. A second wave of environmental exposure is thought to trigger additional changes in the T-cell phenotype and to tip the Th1-Th2 balance, which intensifies the autoimmune attack. When about 80% of the beta-cells are destroyed, insulin secretion is insufficient to maintain glucose homeostasis; then overt diabetes occurs [14, 15].

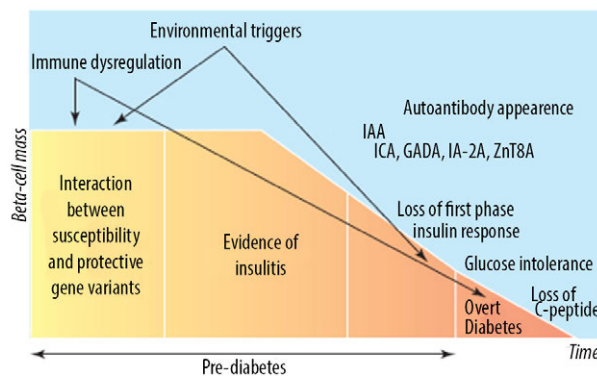


Figure 1 Model of the pathogenesis of T1D. Adapted by permission from BMJ Publishing Group Limited. Devendra et al., 2004 [15].

T1D is usually considered a disease with its onset in childhood, yet about 30% of T1D patients are diagnosed after the age of 30 [16-19]. Compared with T1D children, adult-onset patients usually display less severe symptoms at presentation [20, 21]. In these adult-onset patients, GADA is the most prevalent autoantibody, whereas the decrease in ICA, IA-2A, IAA, and ZnT8A prevalence is more apparent with both increasing age at onset and disease duration [21-26].

In first-degree relatives as well as in the general population, autoantibodies have a high predictive value for T1D. Multiple antibody-positivity is associated with an >65% risk for developing T1D within 5 to 15 years [27-29], whereas single antibody-positivity implies only a small risk, ranging from 3.4 to 4.0% for GADA, ICA, and IAA [30]. Whether autoantibodies also are directly involved in the pathogenesis of T1D is unknown. Compelling evidence exists of the involvement of environmental factors in the pathogenesis of T1D, but despite extensive research, no environmental triggers have conclusively been identified. Thus far, the strongest evidence is for dietary proteins from cow's milk [31, 32] and for early

childhood infections, supported by a possible north-south gradient [8] and seasonality of disease incidence [33, 34]. However, vitamin D exposure, obesity, and environmental pollutants, especially from pesticides, have also been extensively studied but with very conflicting results [9].

1.2. Type 2 diabetes

T2D is estimated to account for 80 to 90% of all cases of diabetes. The prevalence of diabetes among individuals 20 to 79 years of age ranges worldwide from 3.8% in Africa to 8.1% in Europe and 11.7% in North America [35]. The worldwide prevalence of T2D has increased dramatically over the last 30 years, most likely as a consequence of the increased proportion of elderly people and the increased prevalence of obesity.

Even though T2D is far more prevalent than T1D, the aetiology of this diabetic subtype is less-well understood. Its pathogenesis can involve variable degrees of increased insulin resistance and reduced insulin secretion. Whether the disease-causing reduction in insulin secretion is attributable to reduced beta-cell mass or to reduced beta-cell function is, however, unclear [36]. Insulin resistance is a state in which the effect of insulin, the key regulator of blood glucose level, is reduced in target tissues. The main target tissues are skeletal muscles, where insulin induces glucose uptake; the liver, where insulin suppresses the endogenous glucose production; and adipose tissue, where insulin suppresses lipolysis. Because adipose tissue secretes signalling molecules including hormones, cytokines, and free fatty acids, all of which modulate insulin action, insulin resistance is strongly associated with obesity. Age is another factor strongly associated with increasing insulin resistance.

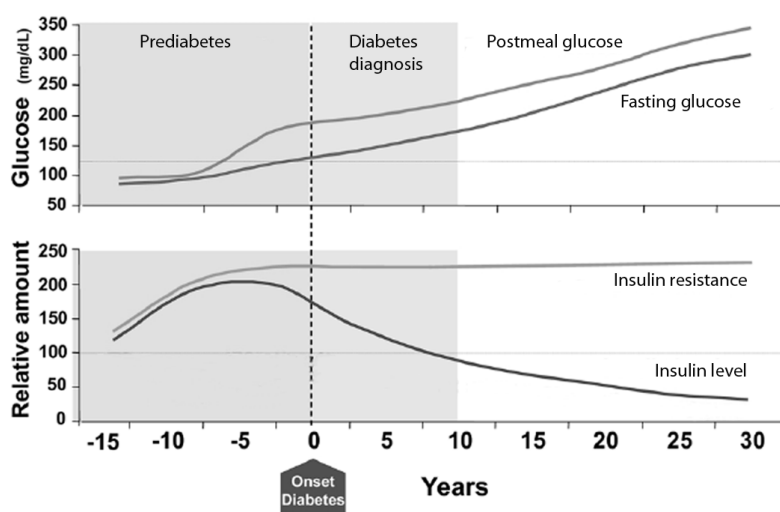


Figure 2 Natural history of T2D. The roles of insulin resistance and of insulin deficiency are highlighted. Adapted from Kendall et al., 2005 [37] with permission from Elsevier.

Figure 2 depicts T2D development in terms of insulin resistance and insulin secretion. Beta-cells adjust insulin production to the state of insulin resistance in order to maintain normo-glycaemia. Development of T2D is a progressive process, in which hyperglycaemia is observed at an early stage as a consequence of insulin resistance or impaired insulin secretion or both. Persistent hyperglycaemia contributes to progressively increasing insulin resistance forcing the beta-cells to compensate by secreting additional insulin. At some point, the beta-cells fail to compensate, and diabetes ensues (Figure 2). The beta-cell potential is individual, and is associated with risk factors for development of T2D, mainly genetic predisposition, family history of diabetes, lifestyle, and intra-uterine environment [38-41].

1.3. The grey zone between type 1 and type 2 diabetes

In clinical practice, diabetes is often classified as T1D or T2D based on phenotypic characteristics including acuteness and age at onset, occurrence of ketoacidosis, need for insulin treatment, and body weight and composition, as well as existence of autoimmune diseases. The classification is, however, not always straightforward. Especially in young and middle-aged adults the difference between T1D and T2D is not clear-cut, and clinical evidence even supports an overlap between T1D and T2D. The two diabetic subtypes cluster in the same families [42]. Family history of T2D is associated with a higher prevalence of T1D compared with prevalence in the general population [43-47], plus later onset, increased prevalence of the metabolic syndrome, increased BMI, and factors related to insulin resistance in T1D patients [48, 49]. Similarly, family history of T1D is associated with increased frequency of T2D [50, 51], and among T2D patients with reduced BMI and C-peptide level, as well as higher prevalence of GADA positivity [52, 53]. Moreover, in initially non-diabetic adults, family history of T1D and GADA are associated with development of non-insulin-dependent diabetes [54]. This familial clustering may indicate that T1D and T2D share disease-underlying genetic and environmental factors.

1.4. Latent Autoimmune Diabetes in Adults - LADA

LADA is a diabetic subtype in the grey zone between T1D and T2D. Among patients of European ancestry initially diagnosed with T2D, LADA accounts for 3 to 11% [55-59]. Its prevalence varies according to population origin, ascertainment, and the diagnostic criteria applied for LADA. LADA diagnosis is based on age at onset, time between diagnosis and initiation of insulin treatment, and presence of autoantibodies [4], of which GADA is the most common type among these patients [60]. Each of these criteria is based on an arbitrary cut-off value, making the diagnosis of LADA unspecific and variable between studies. Table 1 shows the differences in diagnostic characteristics for T1D, LADA, and T2D patients.

Table 1 Diagnostic characteristics of diabetes in adults

	T1D	LADA	T2D
Metabolic syndrome	+	++	+++
Ketoacidosis	+	+/-	-
Complications	+	+	+
Autoantibodies	Positive/negative	Positive (required)	Negative (required)
Age at diagnosis	Usually childhood*	Usually >35	Usually >40
Insulin treatment	At diagnosis	Not at diagnosis	+/-

*About 30% are diagnosed at >30 years.

In the few existing prospective studies on LADA patients, about 50% required insulin after 6 to 10 years of follow-up [55, 61]. However, the clinical phenotype of LADA patients is highly affected by their level of GADA positivity. A high GADA level is associated with reduced beta-cell function, with lower BMI, with lower prevalence of markers of the metabolic syndrome, and possibly with lower age at diagnosis, as well as with an increased insulin requirement [56, 62-65]. In a small study, a high GADA level has further been associated with a higher prevalence of ketosis and diabetic complications [66].

Clinically, LADA appears to be intermediate between T1D and T2D. This notion is based on measures of BMI, lipids, presence of the metabolic syndrome, blood pressure, and prevalence of hypertension [56, 59, 62, 67-72]. Insulin resistance is more pronounced in LADA patients than in T1D patients, whereas it is controversial whether insulin resistance in LADA patients is lower than in T2D patients [56, 67, 73, 74] or similar [69, 75, 76]. This discrepancy may be due to differences in age and BMI between LADA and T2D patients included in the different studies, as both these factors are strongly correlated with insulin resistance. Moreover, in LADA patients compared with T1D patients, the insulin requirement is reduced, and serum C-peptide concentrations are higher [56, 70, 71] and decline at a slower rate, according to some [77], but not all studies [71]. The opposite is observable in comparison of LADA with T2D [55, 56, 77, 78]. LADA patients are therefore considered more insulin deficient than are T2D patients. Risk factors for developing LADA, identified in population-based prospective or cross-sectional studies, are similar to those of T2D and include increased age, over-weight, family history of diabetes, increased waist-hip ratio, stress, and physical inactivity [79-81].

It is evident that diabetes, and in particular LADA, is a heterogeneous disease. This heterogeneity is a reflection of the complex interplay between genes and environment. Improved understanding of factors underlying the disease is the key to improving treatment and potentially preventing diabetes.

2. GENETICS OF DIABETES

An important step in the quest towards understanding mechanisms underlying development of diabetes is to identify the disease-predisposing gene variants, as it is clear that not all

individuals are equally susceptible to the diabetogenic environment of our modern society. Identification of these gene variants can elucidate pathways involved in diabetes pathogenesis and possibly contribute to identification of individuals at increased risk for developing the disease. The range of both effect size and prevalence of variants underlying common diseases is largely unknown and therefore so is the expected number of disease-predisposing variants [82].

Evidence of a genetic component in the pathogenesis of diabetes comes from twin, family, adoption, and admixture studies. Having parents with diabetes imposes an increased risk for diabetes. If the mother has T1D, the child's lifetime risk for T1D is 1 to 3%, and if the father is affected the risk is 4 to 9% [83] compared with only about a 0.4% prevalence in the general population. Having one parent with T2D imposes a lifetime risk of developing T2D of 40%, and if both parents are affected, the lifetime risk is 70%, compared with 10% for the general population [84, 85]. The genetic contribution is also evident from twin studies, where the concordance rate is higher in mono- than in dizygotic twins. For T2D, the concordance rate in monozygotic twins is 35 to 80%, whereas it is 15 to 35% in dizygotic twins [86-90]. For T1D, these rates are 20 to 50% and 5 to 10%. The fact that the concordance rates among monozygotic twins are less than 100% for both T1D and T2D points to an environmental component in disease aetiology as well. Among monozygotic twins discordant for T1D, the risk for T1D developing in the non-affected twin depends on age at diagnosis in the affected twin, with the risk being 38% if the affected twin developed T1D <24 years old, and only 6% if T1D developed >25 years [91]. This variation in risk may indicate a stronger genetic component in early- vs. late-onset T1D.

2.1. The hunt for diabetes susceptibility genes – the pre-GWAS era

Until about five years ago, the genetics of common complex diseases was investigated by linkage analysis or in case-control studies by applying the candidate-gene approach. In linkage analysis, a disease-causing locus is mapped by identifying co-segregation between polymorphic genetic markers and disease in families with multiple affected members [92]. This method has been very successful in detecting rare variants with high penetrance underlying monogenic Mendelian disorders (Figure 3) [93]. But for common complex diseases like T1D and T2D, the success of the linkage approach has been very limited [94]. This lack of success is mostly due to the low penetrance of the risk alleles. A linkage study with enough statistical power to identify a risk locus with an effect size similar to that of known T2D variants would require an extremely large number of families, thus making it impossible to perform. Another problem with linkage studies is their low mapping resolution, making the identification of the causal variant underlying the linkage signal difficult [95-99].

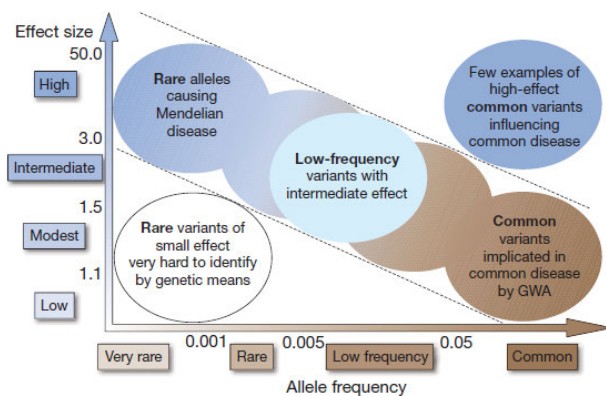


Figure 3 Detectability of disease susceptibility variants depending on effect size and frequency of the risk allele. The diagonal dotted lines mark the focus area of genetic studies. Adapted by permission from Macmillan Publishers Ltd: *Nature* [100], copyright (2009).

Contrary to the linkage approach, a candidate-gene study relies on an *a priori* hypothesis of involvement of the gene in the disease pathogenesis. The candidate genes can be chosen based on knowledge of biological function from genetically engineered animal models or cell studies, based on involvement in monogenic forms of the disease, or based on knowledge of drug targets for the disease [101, 102]. Like linkage analysis, the success of the candidate-gene approach in identifying gene variants underlying common complex diseases has been limited [103]. Reasons for this lack of success include the facts that 1) knowledge of the biological mechanisms underlying most complex diseases, crucial for selection of the candidate gene, is sparse, 2) the case-control study design is vulnerable to population stratification, leading to false positive associations impossible to replicate, and 3) many studies have suffered from small sample size and insufficient phenotyping, reducing penetrance [102, 104]. Sample size is crucial, given that the effect size of common variants associated with common diseases seems to be extremely modest. Hence, a study with a small sample size is limited by its statistical power. With an effect size of 1.3 (odds ratio, OR) and a minor allele frequency <10%, more than 10 000 cases and 10 000 controls are required to obtain a power of 80% to find an association with a convincing p-value [105]. One way to overcome the sample-size problem is to combine studies in a meta-analysis [106]. This study design, however, comes with its own set of potential problems, including publication bias and heterogeneity between study populations [107].

2.2. The hunt for diabetes susceptibility genes – the GWAS era

The advent of genome-wide association studies (GWAS) was a major breakthrough in identification of gene variants underlying complex diseases. This breakthrough was facilitated by advances within several areas. Firstly, the HapMap project [108-110], and later the 1000 Genomes Project [111] contributed with the characterization of single nucleotide

polymorphisms (SNPs), haplotype structure, and linkage disequilibrium (LD) patterns across the genome. LD refers to non-random correlation between alleles at different loci and theoretically allows identification of association with a proxy for the true causal variant without impeding statistical power [112-114]. In practise, this means that it is sufficient to type about 500 000 carefully selected tagSNPs to gain information about the approximately 10 million common variants in the human genome [115, 116]. Secondly, the GWAS were facilitated by development of commercially available, automated, and affordable genotyping technology and advances in statistical methods, as well as collection of sufficiently large sample sets through international collaboration [103].

The contribution of GWAS to knowledge on genetics of common complex diseases is enormous. But despite the large number of susceptibility variants identified for T1D and T2D, genetic prediction of disease development is still poor [117, 118]. Because the GWAS are independent of prior biological knowledge, those susceptibility genes identified have pointed to new T1D- and T2D-associated biological pathways [119, 120]. Biological knowledge will potentially further increase when the causal variants for the remainder of the statistical associations are identified. Genetic information can also prove relevant in motivating patients to change their lifestyle, in identification of possible new drug targets, and in choice of intervention or treatment [121, 122].

The major drawback in GWAS design is the limitation in what is detectable, as depicted in Figure 3. The SNPs on the GWAS genotyping arrays are chosen to be common, with minor allele frequencies >5%. Hence, GWAS are by design only powered to detect association with variants that are common in the population, because the strength of a statistical association between two markers depends on the LD between them. If allele frequencies of the markers differ considerably, the LD between them is low and so is the strength of the association [123]. The common variants identified only explain a fraction of the common disease heritability [100, 117, 124]. Researchers have therefore speculated where to find the remainder of the genetic variation and what methods to apply to find it [100]. Currently, whole-genome and exome sequencing is performed to identify low-frequency variants associated with disease. This type of variation may prove to be of particular importance, because natural selection will maintain deleterious variants at low frequency. This notion is supported by HapMap data showing that rare variants are more likely to be disease-predisposing than are common variants [125]. It is, however, also possible that other types of variation and mechanisms contribute to the heritability of common diseases; these include copy-number variants and gene-gene and gene-environment interactions, as well as epigenetic variability. Thus far, it seems unlikely that copy-number variants will explain additional variation involved in the pathogenesis of either T1D or T2D, as this type of variation is well tagged by SNPs on current GWAS arrays [126]. On the contrary, the contribution of

epigenetics is very plausible and could provide the link between genetic and environmental predisposition.

2.3. Genetics of type 1 diabetes

Currently more than 50 susceptibility loci have been robustly associated with T1D [127]. Knowledge of the genetic predisposition to T1D is primarily based on studies of classical T1D patients diagnosed in childhood or early adulthood, whereas studies of adult-onset T1D are lacking.

Figure 4 displays T1D-susceptibility loci identified up to the year 2009 [128-139]. The candidate-gene approach contributed only five loci identified between 1970 and 2005. Thereafter, the risk loci have been identified by GWAS, or by meta-analyses of GWAS datasets. It is evident from Figure 4 that effect sizes are decreasing as time progresses, attributable to the greater sample size in the most recent studies.

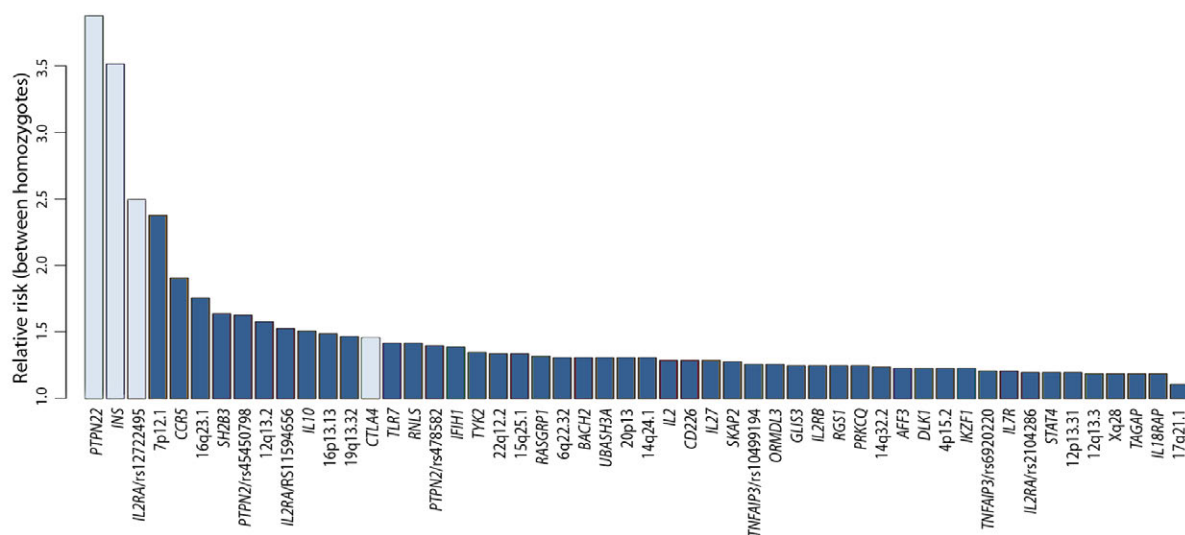


Figure 4 Non-HLA T1D-associated loci identified in candidate-gene studies (light-blue) or GWAS (dark-blue). The loci are represented by the assumed causal gene. Adapted from Todd 2010 [120] with permission from Elsevier.

Estimates are that the identified variants combined explain about 75% of the heritability of T1D [117]. Some of the associated loci lack any compelling functional candidate gene (Figure 4). For others, the strong causal candidate genes identified point to pathways involved in the aetiology of T1D. Among the genes with strong functional support for their involvement in the pathogenesis of T1D are the human leukocyte antigen (HLA) on chromosome 6p21.3, protein tyrosine phosphatase, non-receptor type 22 (*PTPN22*) on chromosome 1p13, insulin (*INS*) on chromosome 11p15, and cytotoxic T-lymphocyte-

associated antigen (*CTLA4*) on chromosome 2q33. These loci are described in the following with key data summarised in Table 2.

The HLA region

The association between HLA alleles and T1D was reported in candidate-gene studies back in the early 1970s [128, 140, 141], and has subsequently been replicated in numerous studies - candidate-gene as well as GWAS - comprising differing ethnic populations [133, 136, 139, 142-144]. With an OR of at least 6, HLA is the major susceptibility locus in T1D and accounts for approximately 40% of its heritability [145].

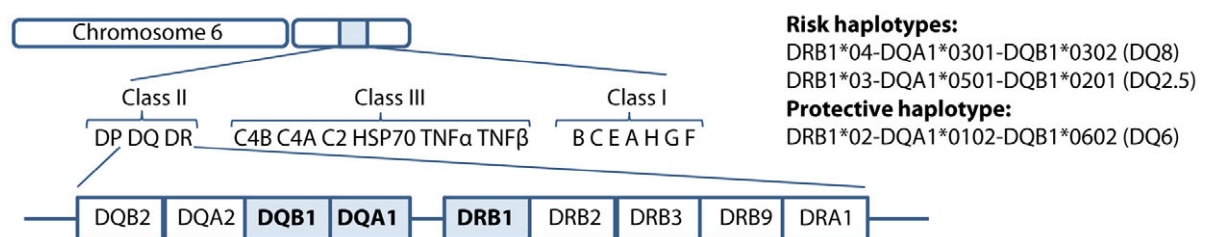


Figure 5 Genes in the HLA region and main T1D-risk and –protective haplotypes.

The HLA region is characterised by a high degree of LD, which complicates assessment of specific disease-associated loci. Figure 5 depicts the three gene subclasses in the HLA region. The primary T1D-risk association is attributed to the HLA-DQ genes and modified by the HLA-DR genes, but association is also indicated for the HLA class I genes, namely HLA-A and HLA-B [142, 143, 146]. In Finland, the DQB1*0302 allele on the DR4 haplotype is associated with the highest risk for T1D, especially when it is co-expressed with the DQB1*0201 allele on the DR3 haplotype [146, 147]. Prevalence of risk-associated alleles varies across Europe. In Finnish T1D children, either or both of these alleles are found in about 90%, compared with their presence in about 40% of Finnish newborns [146, 147]. The DQB1*0602(3) allele on the DR2 haplotype is associated with dominant protection against T1D, and is found in about 36% of Finnish newborns compared with in about 3% of Finnish T1D children [146, 147]. In studies of patients mainly <40 years, prevalence of the high-risk genotypes DQB1*02/*0302 and *0302 (or corresponding DR genotypes), is negatively associated with age at diagnosis of T1D, whereas the protective DQB1*0602 genotype has been positively associated with age at diagnosis [20, 21, 148-154]. Recently, these findings were extended to patients >44 years with autoimmune diabetes [155].

A clear functional link exists between variation in HLA genes and T1D. HLA proteins bind foreign- and self-derived antigens and present them to CD4- and CD8-positive T cells, which activates the immune system and controls T-cell selection [156]. The affinity and

stability of this antigen binding is influenced by variation in *DQB1*; it is hypothesized that susceptibility alleles bind pancreatic antigens less efficiently, which reduces the deleterious signal in autoreactive T-cells, enabling them to escape from negative selection in the thymus [157, 158].

The PTPN22 gene

In 2004, an association between the *PTPN22* C1858T variant and childhood T1D was reported in a candidate-gene study [129]. This association has subsequently been confirmed in GWAS and recently in large meta-analyses comprising up to 16 240 cases and 17 997 control subjects [134, 136, 139, 144, 159]. This association has also been observable in older patients with autoimmune diabetes diagnosed >17 years [155], and in Finnish T1D patients with age at diagnosis between 15 and 40 years [154]. The association between T1D and *PTPN22* has been reported to be stronger in subjects with low-risk HLA genotypes [160, 161] and in GADA-positive T1D patients compared with GADA-negative patients [162].

In 546 Finnish children with T1D (mean age at diagnosis, 8.2±4.1 years) the T-allele of the C1858T variant was observed in 23.9%, whereas its frequency in 538 healthy infants was 13.9% [160]. The frequency both in Finnish cases and control subjects is higher than the frequency observed in other populations [163, 164]. Whether the *PTPN22* variant is associated with age at onset of T1D is controversial. In German T1D children, the T1D-associated T-allele is associated with younger age at onset [165], but no such association appeared in a large UK family-based series [166], in UK T1D children <16 years [161], or in Finnish T1D patients of an age at diagnosis between 15 and 40 years [154].

PTPN22 encodes the lymphoid tyrosine phosphatase (LYP), which functions as a negative regulator of T-cell activation by dephosphorylating T-cell-receptor signalling molecules [167, 168]. The C1858T variant is non-synonymous, changing arginine to tryptophan at position 620 (R620W), and thereby possibly changing the binding properties of LYP. The mechanistic link to T1D is controversial. One suggestion is, however, that the amino acid substitution results in gain of function [169]. Hence, in carriers of tryptophan-containing LYP, inhibition of T-cell receptor signalling rises. In the thymus, this altered signalling can lead to escape of autoreactive T-cells from negative selection and thereby to susceptibility to autoimmune disease [164, 169].

The INS gene

The *INS* locus encoding the insulin protein is an obvious T1D-candidate gene, and association with T1D has been established in children by candidate-gene studies as well as by GWAS [130, 136, 170]. Similarly to *PTPN22*, the association between *INS* and T1D has been reportedly strongest in individuals with low-risk HLA genotypes [171, 172].

The *INS* locus accounts for approximately 10% of the heritability [145]. A high degree of LD characterises the *INS* gene region. Hence, both two SNPs, -23HphI and +1140A/C, and a variable number of tandem repeats (VNTR) located in the *INS* promoter region have been suggested as the causal variant underlying the association with T1D [130, 173]. Expression data have showed that the VNTR is associated with altered insulin transcription [174, 175]. Compared with the class III VNTR (141-209 repeats), the class I VNTR (26-63 repeats) is associated with increased *INS* transcription in the pancreas and decreased transcription in the thymus. This modulated *INS* expression may alter T-cell selection in the thymus and when autoreactive T-cells escape to the periphery reduce tolerance towards insulin [174, 175]. This is a possible functional link between the class I VNTR and predisposition for T1D and is supported by studies showing that insulin is the major initiating antigen in T1D [12, 176]. Interestingly, the association between *INS* and T1D may be modified by epigenetic CpG methylation in the promoter region and may depend on parental origin of the risk allele [177, 178]. In most association studies, the -23HphI SNP is genotyped as a proxy for the VNTR, as the two markers are in near-perfect LD [170].

In Finnish T1D patients diagnosed <35 years, frequency of the T1D susceptibility VNTR class-I allele was 89.2%, whereas the frequency among age- and sex-matched control subjects was 79.5% [179]. This risk-allele frequency among Finns is higher than the frequency in other European subjects [136]. The *INS* variant has also been associated with T1D diagnosed in young Finnish adults (15 to 40 years at diagnosis) [154], and with autoimmune diabetes in German adults (>17 years at diagnosis) [155]. It is controversial whether the variant is associated with age at onset of T1D. In a large family-based series of T1D children, the *INS* variant was associated with older age at onset [166], but with no association evident in analyses of patients diagnosed at over age 15 [154].

The CTLA4 gene

CTLA4 is another T1D susceptibility locus identified in a candidate-gene study in children [131], and subsequently confirmed in GWAS and a meta-analysis comprising up to almost 10 000 cases and 17 000 control subjects [136, 137, 139, 144]. As for the majority of the T1D susceptibility loci, the causal variant underlying the association is yet to be identified; in the *CTLA4* region, the variants most studied are the +49 A/G and the CT60 A/G [180]. For the CT60 variant, an association has also been observable in adults with autoimmune diabetes [155], but no association was observable in Finnish T1D patients diagnosed between ages 15 and 40 [154]. The lack of association in the latter study is possibly due to inadequate sample size, given the small effect size of the *CTLA4* variant (Figure 4). The same study observed no association with age at onset of T1D [154]. Among Finnish T1D children (mean age at diagnosis, 9.8±3.4 years), the T1D-associated G-allele of the *CTLA4* CT60 variant was

evident in 70.4%, whereas the frequency among non-diabetic control subjects was 66.6% [180]. Compared with other Europeans [136, 137], this T1D-susceptibility variant, too, seems to be more common among Finns.

The CTLA4 protein is located on the surface of activated T-cells that produce negative signals for T-cell activation. Functional data suggest that in healthy control subjects, the level of soluble CTLA4 mRNA is significantly correlated with the genotype of the *CTLA4* CT60 variant, possibly explaining the association between that variant and T1D [181].

Table 2 Genetics of T1D – selected variants

Locus	SNP	Variant	Finnish RAF		Factors affecting association	
			Controls	T1D patients	Age at diagnosis	Other
<i>HLA-DQB1</i>	Multiple	*02/*0302	3%	30%	++	
		*02	21%	21%		
		*0302	18%	42%		
<i>PTPN22</i>	rs2476601	Non-synonymous R620W	14%	24%	+/-	HLA genotype GADA positivity
<i>INS</i>	rs689	Non-coding -23HphI	80%	89%	+/-	HLA genotype
<i>CTLA4</i>	rs3087243	Non-coding CT60	67%	70%	-	

RAF, risk allele frequency.

2.4. Genetics of autoantibodies

The gene variants associated with T1D have also been investigated in relation to the prevalence of autoantibodies. In primarily T1D children, HLA-DQB1*0302 (DR4) is positively correlated with IA-2A, IAA, and ICA [23, 30, 155, 182-186], whereas DQB1*02 (DR3) is negatively correlated with IA-2A [23, 155, 184, 186]. The association between HLA genotypes and GADA differs according to diabetes status and age at onset of T1D. In T1D children, GADA is associated with DQB1*02 [23, 30, 187], whereas an association with DQB1*02/*0302 or DQB1*0302 occurs in healthy children [188-190], and in young adults with autoimmune diabetes [155] or with T1D [187, 188].

PTPN22 has been associated with GADA in T1D patients with long disease duration [191] and with IAA in T1D children with HLA-risk genotypes [160]. In contrast, no association between *PTPN22* and IA-2A, GADA, or IAA was observable among 243 German paediatric T1D patients [186]. *INS* has been associated with IAA [23, 192, 193], whereas *CTLA4* does not seem to be associated with T1D-associated autoantibodies [192, 194].

Recently, the genetics of islet autoantibodies (GADA, IA-2A, and ZnT8A) was investigated in GWAS employing >2 200 childhood-onset T1D cases. For GADA, the GWAS supported the primary association with HLA-DR3 (corresponding to HLA-DQB1*02), and further showed an association with an independent SNP in the HLA class I region, *IL2RA* on

chromosome 10p15, and *SH2B3* on chromosome 12q24 [195, 196]. IA-2A was most strongly associated with *HLA-DRB1*, *FCRL3* on chromosome 1q23, *IL27* on chromosome 16p11, *IFIH1* on chromosome 2q24; and further was negatively associated with HLA-A*24 [195, 196]. ZnT8A was associated with *FCRL3* on chromosome 1q23, and the HLA class I region [197]. In the GWAS, no association was observable between GADA, ZnT8A, or IA-2A and the majority of verified T1D-associated loci including *PTPN22*, *INS*, and *CTLA4*, as well as the HLA-DR3/DR4 (*02/*0302) and HLA-DR4 (*0302) genotypes. Hence, based on these data, the genetic predisposition for autoantibodies and T1D seems to differ [195-197].

2.5. Genetics of type 2 diabetes

Currently more than 50 T2D-susceptibility loci are verified in Caucasians [82]. Figure 6 displays the loci identified in candidate-gene studies (n=2) [198, 199], in large-scale association studies (n=3) [200-202], in the first round of GWAS with T2D as the phenotype [133, 203-209], and in a GWAS meta-analysis with a discovery sample comprising 4 549 T2D patients and 5 579 control subjects of European descent (n=13) [210]. The remainder of the validated T2D-risk loci have initially been identified in an even larger meta-analysis [124, 211] and in GWAS of intermediate phenotypes, such as fasting and 2-hour glucose and fasting insulin and proinsulin, as well as measures of insulin resistance and insulin secretion in healthy individuals [212-220].

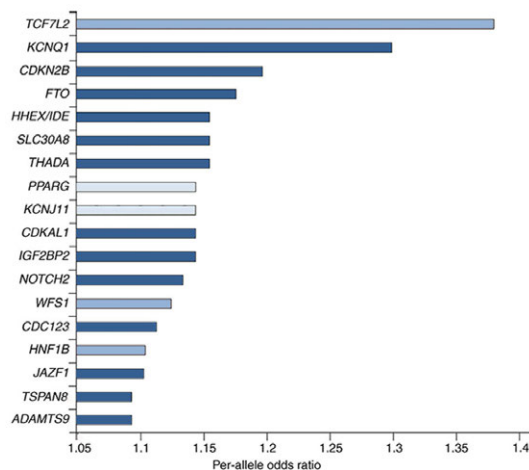


Figure 6 T2D-associated loci identified in candidate-gene studies (light-blue), large-scale association studies (medium-blue), or GWAS (dark-blue). The loci are represented by the assumed causal gene. Adapted from Prokopenko et al. [221] with permission from Elsevier.

It is evident from Figure 6 that the effect sizes of the T2D-risk variants are small. However, the overlap between T2D-susceptibility loci in Caucasian and Asian populations is extensive, suggesting that these associations are explained by causal variants with low effect

sizes in high LD with the identified tagSNPs, rather than causal variants with large effect sizes in low LD with the tagSNP [82, 222].

The annotated T2D-susceptibility genes are inferred from the associated SNPs, of which the majority are located in non-coding regions. Hence, the annotation is tentative, and for most of the loci the causal variant and thereby the direct functional link to T2D is unknown. It is, however, evident that most of the T2D-associated genes identified are involved in beta-cell development, function, or beta-cell mass regulation, including transcription factor 7, like 2 (*TCF7L2*) on chromosome 10q25; solute carrier family 30, member 8 (*SLC30A8*) on chromosome 8q24; and the potassium voltage-gated channel, subfamily Q, member 1 (*KCNQ1*) on chromosome 11p15. Few genes that are involved in insulin resistance have been identified, including peroxisome proliferator-activated receptor γ (*PPARG*) on chromosome 3p25, and fat mass and obesity associated (*FTO*) on chromosome 16q12 [221,223, 224]. These loci are described in the following with key data summarised in Table 3.

The TCF7L2 gene

Based on a linkage study suggesting association with T2D on chromosome 10q [225], a large-scale association study of micro-satellites identified the *TCF7L2* rs7903146 variant [200]. This intronic variant is thus far the strongest genetic risk factor for T2D, with an OR >1.3, and its association with T2D has been replicated in numerous studies and in different ethnic populations [226, 227]. In non-diabetic Finnish subjects, the frequency of the T2D-associated rs7903146 T-allele has been reported to be 18% [206, 228], lower than the frequency observed in other European subjects [229].

In population-based samples and in healthy subjects, the rs7903146 T-allele has been associated with an increased pro-insulin-to-insulin ratio, with reduced insulin secretion, with an enhanced rate of hepatic glucose production, and with impaired incretin effects [228, 230-233]. The exact mechanistic link between the *TCF7L2* variant and reduced beta-cell function is, however, still unclear. The *TCF7L2* protein product is a transcription factor involved in the WNT-signalling pathway. In islets from T2D patients carrying the T2D-associated T-allele of the rs7903146 variant, *TCF7L2* transcription is increased [230]. And overexpression of *TCF7L2* in human islets has been associated with reduced glucose-stimulated insulin secretion [124, 230]. The T-allele has also been associated with a more open chromatin state in human islets and with greater enhancer activity [234]. The chromatin state may signify increased transcription of *TCF7L2*. Together, these studies provide a possible functional link for the intronic variant.

The SLC30A8 gene

The *SLC30A8* R325W variant was identified as a T2D-susceptibility variant in the first published T2D GWAS [203], and has been confirmed in subsequent studies [235]. In Finnish non-diabetic subjects, the frequency of the T2D-associated C-allele was 61% [206, 228], which is lower than the frequency observed in non-diabetic UK subjects [205].

In normal glucose-tolerant subjects, the T2D-associated C-allele of the R325W variant is associated with traits related to beta-cell function, ones such as conversion of pro-insulin to insulin [219, 236], reduced insulin release, and first-phase insulin response [124, 204, 228, 237, 238]. Even though no effect of the variant was observable on basal or stimulated insulin secretion in human islets *in vitro* [235], R325W is most likely the causal variant, and strong evidence exists for the underlying mechanism. *SLC30A8* encodes the zinc transporter type 8 (ZnT8); this protein is islet-specific and facilitates proper storage and secretion of insulin [239], and it is an example of a possible new drug target identified based on genetic information [240]. Drug-mediated enhancement of the intracellular function of the zinc transporter could potentially improve insulin secretion. Its potential as drug target is supported by studies showing that ZnT8 downregulation in INS-1 cells reduces glucose-stimulated insulin secretion, whereas ZnT8 overexpression had the opposite effect [239, 241]. ZnT8 also provides an interesting link between T2D and T1D, because the protein is the target of the ZnT8 autoantibodies, and the R325W variant determines antigen specificity in T1D patients but is not associated with T1D [242, 243].

The KCNQ1 gene

Two independent Japanese GWAS identified *KCNQ1* as a T2D-susceptibility gene, an association subsequently replicated in Europeans [124, 208, 209]. This association was missed in the first round of European GWAS, illustrating the importance of considering differing ethnic groups when mapping genetic associations. In population-based Finnish samples, the T2D-associated C-allele of the *KCNQ1* rs2237895 variant was observable in 47 to 49% [244]. This is higher than the frequency observed in Danish subjects with normal glucose tolerance [208]. *KCNQ1* encodes the pore-forming alpha subunit of the $I_{Ks}K^+$ channel expressed in the pancreas [208, 209], making it a good T2D candidate gene. Following an oral or intravenous glucose load in glucose-tolerant individuals, the T2D-associated C-allele of the rs2237895 variant is associated with lower glucose-stimulated insulin secretion [124, 244-246], but not with *KCNQ1* expression in human islets *in vitro* [244].

The PPARG gene

PPARG is the first confirmed T2D-susceptibility gene. This association was established in a candidate-gene study [198], based on knowledge of the receptor's being a target for the T2D

thiazolidinedione drugs [247]. The association has subsequently been confirmed in GWAS and in a large meta-analysis comprising 32 849 T2D cases and 47 456 control subjects [205-207, 248]. In non-diabetic Finnish subjects, the *PPARG* rs1801282 T2D-associated C-allele was observable in 82 to 85% [206, 228], which is similar to the C-allele frequency in other European populations [248].

The risk variant is non-synonymous, changing proline to alanine at position 12 (P12A), and has been associated with decreased transcriptional activity and increased insulin resistance [124, 249]. The *PPARG* protein is a transcription factor primarily expressed in adipose tissue, where it regulates adipocyte differentiation, fatty acid storage, glucose metabolism, insulin sensitivity, and inflammation [250-252].

The FTO gene

FTO was initially identified as an obesity-susceptibility locus [253, 254] and subsequently as a T2D-associated locus [205, 254, 255] providing a molecular link between obesity and T2D. The association between the *FTO* rs9939609 intron variant and T2D was initially considered BMI-dependent, but two large studies have recently showed that the association remains after adjustments for BMI and other anthropometric measures [255, 256]. In a population-based Finnish sample, the frequency of the T2D-associated A-allele of the rs9939609 variant was 39% [257], similar to the frequency in UK control subjects [205].

The molecular mechanism underlying the association is unclear, as the function of the ubiquitously expressed *FTO* gene product is unknown. In non-diabetic subjects and in population-based samples, the T2D-associated *FTO* variant has been associated with peripheral insulin resistance [124, 258-261], which is dependent on BMI in some [258-260] but not all studies [261]. In mice, systematic *Fto* overexpression results in increased energy intake and increased adiposity, whereas *Fto* knockout results in reduced fat mass [262, 263].

Table 3 Genetics of T2D – selected variants

Locus	SNP	Variant	RAF	Function	Mechanistic link
<i>TCF7L2</i>	rs7903146	Intronic	18%	Transcription factor involved in glucagon and insulin secretion	Beta-cell function
<i>SLC30A8</i>	rs13266634	Non-synonymous R325W	61%	Zinc transporter in beta cells	Beta-cell function
<i>KCNQ1</i>	rs2237895	Intronic	47-49%	Ion-channel subunit	Beta-cell function
<i>PPARG</i>	rs1801282	Non-synonymous P12A	82-85%	Transcription factor involved in adipocyte function	Insulin resistance
<i>FTO</i>	rs9939609	Intronic	39%	Unknown	BMI, insulin resistance

RAF, risk allele frequency (in Finnish non-diabetic subjects).

2.6. Genetic overlap between type 1 and type 2 diabetes

The best piece of evidence for a common genetic predisposition for T1D and T2D is the *GLIS3* locus. Different SNPs in high LD within this locus have been associated with T1D in Europeans [138, 139], with T2D in Asians [264, 265], and in non-diabetic subjects associated with elevated fasting plasma glucose as well as reduced beta-cell function [217, 266, 267]. *GLIS3* is a strong candidate gene for predisposition to diabetes, as it encodes a Krüppel-like zinc finger transcription factor highly expressed in beta cells. The transcription factor is proposed to be a key player in the regulation of beta-cell development and in insulin gene expression [268, 269]. Another possible genetic link between T1D and T2D is the *PPARG* locus. The association between the P12A variant in this gene and T2D is well-established [248], but the variant has also been associated with T1D in well-powered studies of up to 7 606 T1D children [270-272].

Based on the other common T1D- and T2D-susceptibility gene variants, very little evidence exists of a direct genetic overlap between the two diabetic subtypes. A large meta-analysis comprising more than 4 000 T2D cases and more than 5 000 control subjects reported no convincing associations with nine T1D-susceptibility loci, including *PTPN22* and *CTLA4* [273]. Similarly, no association was observable between the *INS* VNTR and T2D in a study comprising 1 462 patients and 4 931 control subjects [274]. In large studies of T1D children with a statistical power of at least 80% to detect association with an OR of 1.11, conflicting results have emerged for *THADA* and *HHEX* [137, 270, 275], whereas no association was observed for other common T2D loci, including *TCF7L2*, *FTO*, and *SLC30A8* [243, 270, 275-278]. Furthermore, no association has been observed between age at onset of T1D and any of the T2D-susceptibility variants [270, 275-278].

In line with this, neither was any association observable between variants in 11 T2D-susceptibility genes, including *FTO*, *KCNQ1*, *PPARG*, *SLC30A8*, and *TCF7L2*, and development of islet autoantibodies in 1 350 prospectively-followed children with T1D parents [279]. And 1 212 patients with autoimmune diabetes showed no association with *TCF7L2* or *FTO* [155]. The apparent lack of genetic overlap between T1D and T2D is, however, based on current knowledge of genetic susceptibility, which is limited to common variants; it is therefore possible that yet-unidentified variants will alter this picture.

2.7. Genetics of LADA

Current knowledge of the genetic predisposition to LADA is mainly based on small candidate-gene studies focusing on known T1D- or T2D-susceptibility variants. Only six of these studies comprise more than 100 LADA patients, and are summarised in Table 4. The general small sample size and heterogeneity in LADA have resulted in conflicting results.

Hypothesis-free studies of the genetic background for LADA are lacking, leaving the possibility that LADA has a unique genetic background yet unexplored.

Table 4 Summary of major LADA studies

Study	Origin	n LADA	LADA selection criteria				References
			Age at diagnosis	Insulin free (months)	Autoanti-bodies	Clinical diagnosis	
Botnia	Finland	104	No criterion*	≥6	GADA	T2D	[56]
HUNT	Norway	126	No criterion	≥12	GADA	Not reported	[65]
Skåne Diabetes Register	Sweden	164	>35 years	No criterion	GADA	Not reported	[280]
Semmelweis	Hungary	211	>35 years	≥6	GADA/ICA/IA-2A/IAA	Not reported	[281]
NIRAD	Italy	193/250	No criterion	≥6	GADA	T2D	[62, 282, 283]
UKPDS [‡]	UK	378/400	≥25 years	≥3	GADA/IA-2A	T2D	[284, 285]

*95% of the LADA patients were diagnosed at >35 years; these patients (n=99) were included in Studies I and III. [‡]Collected from 3 different sources.

Of the T1D-susceptibility gene variants, consistent association with LADA has been apparent for HLA, especially HLA-DQB1*02/*0302 [56, 62, 65, 280, 285]. The frequency of the HLA-risk genotypes is lower among LADA patients than among T1D patients who are diagnosed primarily at <35 years, according to some [56, 65] but not all studies [280]. The main protective DQB1 allele, *0602(3), is more frequent in LADA than in T1D patients [56, 65, 280], and has been less [62, 65, 280, 285] or equally [56] frequent when compared with its occurrence in T2D or control subjects. Within LADA, the frequency of the HLA-risk and -protective alleles varies according to GADA level [56, 62, 65]. The *PTPN22* C1858T variant has been associated with LADA in general [280] or with LADA with high GADA levels [282]; this association is, however, not supported by recent findings [65]. Moreover, data on the T1D-associated *INS* VNTR have been conflicting: some studies show association between the variant and LADA [280, 284], whereas others show none [56, 65]. Only one study, including more than 100 LADA patients, has assessed the effect of *CTLA4* and found no association [65].

With respect to the T2D-susceptibility gene variants, results are few and conflicting. *TCF7L2* has been associated with LADA in general [280, 281], with LADA with BMI <25 kg/m² [281], with LADA with low GADA levels [283], or no association with LADA has been observable [65]. In addition, the *TCF7L2* variant distinguishes middle-aged antibody-positive patients from young antibody-positive patients [286]. Regarding other T2D-susceptibility variants, an association has been suggested for *FTO* and *TSPAN8* mainly in

LADA patients with low GADA levels, whereas no association is apparent for *SLC30A8*, *PPARG*, or *KCNQ1* [65]. Table 5 summarises current knowledge of LADA genetics.

Table 5 Genetics of LADA

Locus	Variant	Association	
		LADA	GADA level
T1D-associated loci			
<i>HLA-DQB1</i>	*02/*0302	+	+
<i>PTPN22</i>	C1858T	+/-	+
<i>INS</i>	VNTR	+/-	
<i>CTLA4</i>	CT60	(-)	
T2D-associated loci			
<i>TCF7L2</i>	rs7903146	+/-	+
<i>FTO</i>	rs8050136	(+)	+
<i>TSPAN8</i>	rs7961581	(+)	+
<i>SLC30A8</i>	R325W	(-)	
<i>PPARG</i>	P12A	(-)	
<i>KCNQ1</i>	rs2237895	(-)	

Loci associated with LADA and relevant loci showing no association. Parentheses indicate an observation reported by only one study. Only results from studies including more than 100 LADA patients are included.

Increased knowledge of the genetic background for LADA is of great importance, because such data can provide clues to its underlying pathogenesis and determine whether LADA really is a subtype of T1D or rather a unique disease entity in the grey zone between T1D and T2D.

AIMS OF THE STUDY

To compare patients with adult-onset T1D and LADA with respect to

- clinical characteristics, namely insulin secretion and metabolic traits
- prevalence and phenotypic associations of ZnT8A
- association with T1D- and T2D-susceptibility gene variants

To investigate the genetic heterogeneity in LADA related to

- GADA level
- family history of T1D

To assess the associations of T1D-susceptibility gene variants with

- phenotype of LADA patients
- insulin secretion and GADA positivity in non-diabetic adults
- progression to non-insulin-dependent diabetes

SUBJECTS AND METHODS

1. STUDY POPULATIONS

All studies were approved by locale ethics committees and all study subjects gave informed consent.

1.1. The Botnia Study

The Botnia Study was initiated in 1990 with the goal of identifying genes underlying metabolic defects and T2D [287]. All known T2D patients and their family members from the health care districts of Malax, Korsholm, Närpes, Jakobstad, and Vasa were invited to participate in the study. In 1994 the study was expanded phenotypically, to include also T1D patients, and geographically, to include patients from other parts of Finland and southern Sweden. In total, the Botnia Study includes 11 000 subjects from 1 400 families.

1.2. The Botnia Prospective Study

In the Botnia Prospective Study, initially non-diabetic family members and spouses (with no family history of diabetes) of the T2D patients from the Botnia Study were invited to follow-up visits every 3 to 5 years [38, 54]. By 2009, about 2 800 individuals had participated in at least one follow-up examination. At baseline, 2 029 (73.4%) showed normal glucose tolerance, and 735 (26.6%) showed impaired fasting glucose or impaired glucose tolerance. The subjects were followed for a mean time of 8.1 years. Data on first- to third-degree family history of type 1 and type 2 diabetes was obtained through questionnaires or clinical investigation of family members.

1.3. The Botnia Mix Study

The Botnia Mix Study was set up to assess impact of family history of T1D on the phenotype of T2D patients. Since 2002, T2D patients with age at onset above 20 years, no insulin treatment within the first year of diagnosis, and with first- to third-degree relatives with T1D have been recruited through newspaper advertisements and via diabetes nurses around Finland. The total of 196 T2D patients included were interviewed by the study physician. The diagnosis of T1D among their relatives was verified by clinical investigation or through interview and/or patient records.

1.4. The FinnDiane Study

The Finnish Diabetic Nephropathy (FinnDiane) Study is nationwide and prospective, initiated in 1997 to identify genetic and clinical risk factors for diabetic nephropathy in patients with

T1D [68]. The study comprises over 4 500 T1D patients, collected from more than 70 centres across Finland.

2. STUDY SUBJECTS

Diabetes was diagnosed as fasting plasma glucose concentration ≥ 7.0 mmol/l or 2-hour plasma glucose concentration ≥ 11.1 mmol/l in compliance with WHO recommendations. T1D was clinically diagnosed and required a fasting C-peptide concentration of < 0.2 nmol/l at the time of investigation, and an initiation of insulin treatment within 6 months of diagnosis. The LADA patients were clinically diagnosed with T2D and were additionally required to be over age 35 at the time of diagnosis, GADA positive, and treated without insulin for the first 6 months after diagnosis. T2D was clinically diagnosed, and all T2D patients were GADA-negative.

2.1. Study I

This study comprised patients from the Botnia Study with LADA (n=213), adult-onset T1D (> 35 years at diagnosis; n=35), young-onset T1D (< 20 years at diagnosis; n=158), and T2D (n=648), as well as 710 non-diabetic control subjects with no family history of diabetes. Additionally included were 222 adult-onset T1D patients from the FinnDiane Study.

2.2. Study II

This study comprised 2 764 initially non-diabetic spouses or relatives of T2D patients from the Botnia Prospective Study who had follow-up data available. After a mean follow-up time of 8.1 ± 3.6 years, 170 (6.2%) had developed diabetes (T1D, n=3; T2D, n=133; LADA, n=34), and 253 (9.2%) were GADA-positive.

2.3. Study III

This study comprised patients with LADA (n=294; 312 from the Botnia Study, and 5 from Helsinki University Central Hospital) and with type 1 diabetes diagnosed after the age of 35 (n=274; 29 from the Botnia Study, 222 from the FinnDiane Study, and 23 from Helsinki University Central Hospital), as well as non-diabetic control subjects with no family history of diabetes (n=537, from the Botnia Study).

2.4. Unpublished data

From the Botnia Mix Study, 196 patients with a family history of both T1D and T2D (LADA, n=28; T2D, n=168), 139 patients (LADA, n=6; T2D, n=133) matched for age, gender, BMI, and disease duration with a T2D family history only, and 274 patients from the Botnia Study (LADA, n=188; T2D, n=86) with a family history of T1D or T2D or both underwent study.

3. METABOLIC MEASUREMENTS AND ASSAYS

3.1. Oral glucose tolerance test (OGTT)

To estimate glucose tolerance, all non-insulin-dependent study subjects underwent an oral glucose tolerance test (OGTT), in which 75 g of glucose was administered after an overnight fast. Blood samples were drawn at -5, 0, 30, 60, and 120 minutes for measurement of glucose, insulin, and C-peptide concentrations.

3.2. Basic measurements

BMI was calculated based on measures of body weight and height as weight in kilograms divided by height in meters squared. Waist-hip ratio was calculated from waist circumference, measured at the midpoint between the iliac crest and the lowest rib, and hip circumference, measured at the widest part of the gluteal region. Systolic and diastolic blood pressure were determined as the mean of two measurements.

Plasma glucose was measured with a glucose oxidase method applying a Beckman Glucose Analyzer II (Beckman Instruments, Fullerton, CA, USA). Serum insulin concentrations were measured either by RIA (Pharmacia, Uppsala, Sweden), with an interassay CV of 5%; ELISA (DAKO Diagnostics Ltd, Cambridgeshire, UK), with an interassay CV of 8.9%; or with the AutoDelfia immunofluorometric assay (Perkin Elmer Finland, Turku, Finland). Insulin concentrations obtained with the RIA and AutoDelfia methods were transformed to cohere with ELISA measurements. The transformation factors were obtained from a parallel analysis of insulin concentrations of duplicate serum samples from 400 subjects. The correlation coefficient between ELISA and both RIA and AutoDelfia was 0.98 ($p < 0.0001$). Serum C-peptide concentrations were determined by the RIA radioimmunoassay with an interassay CV of 9% (Human C-peptide RIA; Linco, St. Charles, MO, USA). A Cobas Mira analyser (Hoffman LaRoche, Basel, Switzerland) was applied to measure total cholesterol, HDL-cholesterol, and triglyceride concentrations in fasting serum samples. HbA_{1c} concentrations were measured by high-pressure liquid chromatography with a reference value of 5 to 7%.

Insulin sensitivity was evaluated by the HOMA insulin resistance index ($HOMA_{IR} = fS\text{-insulin} \times FPG / 22.5$) [288] or the insulin sensitivity index ($10,000 / \sqrt{(FPG \times fS\text{-insulin} \times \text{mean OGTT}_{\text{glucose}} \times \text{mean OGTT}_{\text{insulin}})}$) [289]. Beta-cell function was estimated by OGTT data as the insulinogenic index ($(\text{insulin}_{30} - \text{insulin}_0) / (\text{glucose}_{30} - \text{glucose}_0)$) or corrected insulin response ($CIR, 100 \times \text{insulin}_{30} / (\text{glucose}_{30} \times (\text{glucose}_{30} - 3.89))$) [290].

3.3. Autoantibody assays

GADA, ZnT8A, and IA-2A were measured in fasting serum samples with radiobinding assays using ^{35}S -labelled protein (GAD65, IA-2ic, or ZnT8) produced by coupled *in vitro* transcription-translation as described earlier [56, 291].

The GADA concentration was expressed as relative units (RU) until the year 2000, and thereafter as international units/ml (IU/ml) according to the WHO international standard. The positive cut-off limit was determined as the mean+3 SD in 296 healthy Finnish control subjects, corresponding to 5 RU or 32 IU/ml in assays employing the pEx9 plasmid and 50 IU/ml in assays employing the pThGAD65 plasmid (after 2008). The two different scales of positive values prevented direct analysis of the GADA level in the whole patient material; instead the level was analysed separately for RU and IU/ml units based on quartiles (lowest, 5 to 7.5 RU or 32 to 43.9 IU/ml; middle, 7.6 to 96.9 RU or 44 to 278 IU/ml; highest, >96.9 RU or >278 IU/ml) or the median (18.7 RU or 80.7 IU/ml). In autoantibody workshops (Combinatorial or the Diabetes Autoantibody Standardization Program, DASP) between 1998 and 2005, the GADA assay showed 82 to 84% sensitivity and 95 to 96% specificity [292].

The ZnT8A_{RW} assay employed the chimeric recombinant plasmid (provided by J. Hutton, University of Colorado, Denver, USA) containing the C-terminal part (aa 268-369) of ZnT8-325R and ZnT8-325W for initial ZnT8A screening. ZnT8A_R and ZnT8A_W reactivity was measured in ZnT8A_{RW}-positive samples. ZnT8A concentrations were expressed as RU derived from a standard curve of pooled strongly ZnT8A-positive samples diluted in normal human serum. The positive cut-off limits were 0.61 RU (ZnT8A_{RW}), 1.97 RU (ZnT8A_R), and 3.40 RU (ZnT8A_W), corresponding to the 99th percentile in a combined sample of non-diabetic Finnish children and adolescents (n=250) as well as the DASP control panel (n=100). In the 2009 DASP workshop, all three ZnT8A assays showed 100% specificity and 63% (ZnT8A_{RW}), 48% (ZnT8A_R), and 34% (ZnT8A_W) sensitivity [293].

IA-2A concentration was expressed as RU with a positive cut-off limit of 2.5 RU, representing the mean+3 SD in 155 healthy Finnish control subjects. In DASP workshops between 2002 and 2005, the assay showed 62 to 70% sensitivity and 99 to 100% specificity [292].

4. GENOTYPING

To assess the contribution of T1D- and T2D-susceptibility gene variants in LADA and adult-onset T1D, we genotyped variants chosen based on effect size and knowledge of validation in Finns and other European populations at the time when the study was planned. The contribution of T1D susceptibility was represented by variants in *HLA-DQB1*, *PTPN22*, *INS*, and *CTLA4*. The contribution of T2D-susceptibility genes affecting beta-cell function was

represented by variants in *TCF7L2*, *SLC30A8*, and *KCNQ1*; and T2D-susceptibility genes affecting insulin resistance were represented by variants in *PPARG* and *FTO*.

4.1. *HLA-DQB1* automated method (Studies I, III, and unpublished data)

Applying the automated method [294], the second exon of *HLA-DQB1* was PCR amplified and subsequently hybridized with lanthanide (III) chelate-labelled DNA probes specific for the *HLA-DQB1**02, *0301, *0302, *0602, and *0603 alleles. Hybridization was evaluated by time-resolved fluorescence (Delfia Research Fluorometer, Wallac OY, Turku, Finland).

4.2. Taqman allelic discrimination (HLA: Study II; other SNPs: Studies II, III, and unpublished data)

Genotyping of variants in *PTPN22* (rs2476601), *INS* (rs689), *CTLA4* (rs3087243), *HLA-DQA1-DQB1* (rs2187668, tagging *DQA1**05/*DQB1**02 (DQ2.5), and rs7454108 tagging *DQA1**03/*DQB1**0302 (DQ8)), *TCF7L2* (rs7903146), *SLC30A8* (rs13266634), *KCNQ1* (rs2237895), *PPARG* (rs1801282), and *FTO* (rs9939609) was carried out by fluorogenic 5' nuclease allelic discrimination chemistry (TaqMan®) on an ABI Prism® 7900 Sequence Detection System (Applied Biosystems, Foster City, CA, USA). The assay mix containing primers and probes was designed by Applied Biosystems.

4.3. Genetic risk scores

Study I

The combined effect of the T1D-susceptibility variants in *HLA-DQB1*, *PTPN22*, *INS*, and *CTLA4* was assessed as a genetic risk score. The number of risk genotypes (*HLA-DQB1**02/*0302 and *0302/X, *INS* AA, *PTPN22* CT/TT, and *CTLA4* GG) was counted (0-4), and subjects having 0, 1, or ≥ 2 risk genotypes were grouped. Subjects with one or more missing genotypes (n=33) were excluded from analysis.

Study II

The T1D-susceptibility variants in *HLA-DQ*, *PTPN22*, *INS*, and *CTLA4* were combined in a genetic risk score where each variant contributed in correspondence to its level of effect size. DQ2.5/DQ2.5, DQ2.5/DQX, *INS* AA, *PTPN22* CT/TT, and *CTLA4* GG each contributed with one, DQ8/DQ8 and DQ8/DQX with two, and DQ2.5/DQ8 with three points to the risk score. For the analyses, subjects were divided into groups having a genetic risk score (0-6) of ≤ 1 , 2 to 3, or ≥ 4 . Subjects with one or more missing genotypes (n=65) were excluded from analysis.

5. STATISTICAL ANALYSES

All statistical analyses were performed with the Statistical Package for Social Science software (SPSS, Chicago, IL, USA). A p-value <0.05 was considered significant. All p-values presented are uncorrected for multiple testing. Differences in genotype distributions as well as GADA and ZnT8A prevalence were analysed with the chi-squared test or logistic regression. Differences in quantitative traits including ZnT8A level were assessed with the Mann-Whitney U-test or a general linear model after logarithmic transformation when appropriate. A Cox regression analysis was applied to assess the risk of developing non-insulin-dependent diabetes from baseline to follow-up.

RESULTS

1. CLINICAL COMPARISON OF ADULT-ONSET T1D AND LADA (Studies I and III)

Adult-onset T1D patients diagnosed above the age of 35 years (T1D_{>35}) were similar to T1D patients diagnosed before age 20 (T1D_{<20}) regarding metabolic traits (BMI, triglycerides as well as HDL- and LDL-cholesterol) and level of hyperglycaemia (HbA_{1c}; Figure 7), despite large differences in age (at diagnosis, mean±SD: 41.8±6.6 vs. 10.0±5.0 years; at investigation, 53.5±8.7 vs. 33.6±13.7 years) and duration (11.7±7.9 vs. 23.7±13.0 years).

LADA patients' figures were intermediate between those of T2D and T1D_{>35} patients both metabolically and with respect to level of hyperglycaemia (HbA_{1c}: LADA vs. T2D, 7.9±0.2% vs. 7.4±0.1%, $p=0.01$; vs. T1D_{>35}, 8.5±0.1%, $p<0.0001$; Figure 7). The beta-cell function also differed between patient groups; the level of insulin secretion was significantly lower in LADA than in T2D patients (fS-C-peptide, 0.49±0.03 vs. 0.67±0.02 nmol/l, $p<0.00001$; Figure 7), but was significantly higher than in T1D_{>35} patients ($p<0.00001$), whose fS-C-peptide by criterion was <0.2 nmol/l.

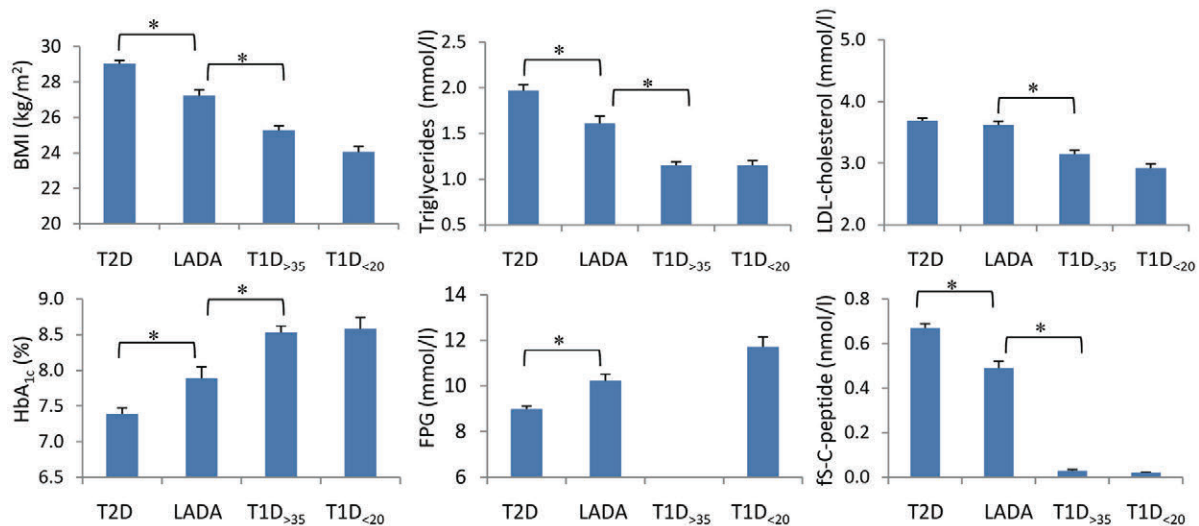


Figure 7 Clinical characteristics of patients with T2D, LADA, T1D_{>35} (FPG data were not available), and T1D_{<20}. Data are mean values (SEM). * BMI, LADA vs. T2D/T1D_{>35}: $p<0.0001/p<0.001$; HbA_{1c}, $p=0.01/p<0.0001$; FPG, $p<0.0001$; triglycerides, $p<0.00001$; LDL-cholesterol, $p<0.00001$; fS-C-peptide, $p<0.00001$. p -values obtained with a general linear model adjusted for age (Study I).

LADA and T1D_{>35} patients also differed regarding prevalence of ZnT8A. Hence, ZnT8A were observed more often in LADA patients than in T1D_{>35} patients (ZnT8A_{RW}: 34.3% vs. 18.7%, $p<0.0001$; ZnT8A_R: 26.9% vs. 8.0%, $p=0.0002$; ZnT8A_W: 26.1% vs. 6.1%, $p<0.00001$). Among ZnT8A-positive subjects, the autoantibody levels in LADA and T1D_{>35} patients were similar (data not shown). In T1D_{>35} patients, ZnT8A prevalence was negatively correlated with disease duration ($p<0.0001$; Figure 8), and ZnT8A specificity was associated with *SLC30A8* R325W genotype; the CC genotype of the *SLC30A8* R325W variant was associated with presence of ZnT8A_R (*SLC30A8* CC vs. CT vs. TT: 16.1% vs. 2.5% vs. 6.5%, $p=0.001$), whereas the TT genotype was associated with the presence of ZnT8A_W (*SLC30A8* CC vs. CT vs. TT: 3.4% vs. 3.4% vs. 17.4%, $p=0.001$; data not shown). Among LADA patients, the ZnT8A prevalence was not associated with disease duration (Figure 8), nor was ZnT8A specificity associated with *SLC30A8* R325W genotype (data not shown).

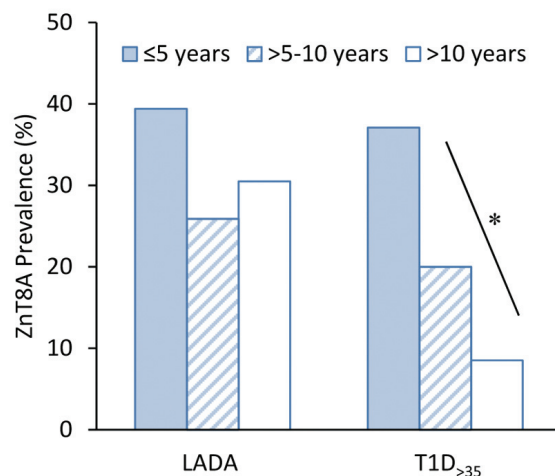


Figure 8 Prevalence of ZnT8A according to duration in LADA (n: ≤5 years, 142; >5-10 years, 58; >10 years, 82) and in T1D_{>35} (133, 93, and 36) patients. * $p<0.0001$, assessed with the Chi-squared test (Study III).

ZnT8A positivity was not a major phenotypic determinant in LADA or T1D_{>35}. ZnT8A were associated with older age at diagnosis among LADA patients (ZnT8A-positive vs. -negative, 60.0 (17.0) vs. 53.0 (16.3) years, $p=0.002$), but not with reduced insulin secretion or insulin sensitivity in either LADA or T1D_{>35} patients (data not shown). Among LADA patients, ZnT8A positivity in combination with a high GADA level (>median) signified a strong autoimmune profile, which was associated with reduced insulin secretion (fS-C-peptide, $p<0.0001$) and lower BMI ($p=0.011$; Table 6).

Table 6 Clinical characteristics by autoimmune profile in LADA patients

	Autoimmune profile		p-value
	Strong	Weak	
n	45	88	
Age at diagnosis (years)	51.0 (19.5)	53.5 (17.8)	0.614
BMI (kg/m ²)	25.73 (4.41)	27.53 (5.19)	0.011
Duration (years)	4.0 (10.8)	6.0 (10.0)	0.345
FPG (mmol/l)	8.8 (5.1)	8.1 (5.9)	0.734
Insulinogenic index	2.83 (3.94)	4.87 (10.15)	0.257
CIR	26 (41)	34 (70)	0.178
fS-C-peptide (nmol/l)	0.30 (0.46)	0.61 (0.51)	<0.0001

Data are median (interquartile range). Differences between patients with a strong (GADA >median and ZnT8A+) and a weak (GADA <median and ZnT8A-) autoimmune profile were assessed with the Mann-Whitney U-test (Study III).

2. GENETIC COMPARISON OF ADULT-ONSET T1D AND LADA (Studies I, III, and unpublished data)

The T1D-risk variants were associated with both T1D_{<20} and T1D_{>35}. Compared with the frequency in non-diabetic control subjects, *HLA-DQB1*-, *PTPN22*-, *INS*-, and *CTLA4*-risk genotypes were significantly more frequent in both T1D_{<20} and T1D_{>35} patients (*HLA-DQB1**02/*0302 or *0302/X, $p<0.0000001$ for both; *PTPN22* CT/TT, $p=0.006$ and $p<0.000001$; *INS* I/I, $p=0.0002$ and $p<0.000001$, *CTLA4* GG, $p=0.008$ and $p=0.003$). The *HLA-DQB1*-protective genotypes were equally rare in both T1D_{<20} (3.8%) and T1D_{>35} patients (2.3%), and the frequency in both groups of T1D patients differed significantly from their frequency in non-diabetic control subjects (*0602(3)/X or *0602(3)/*0301, $p<0.0000001$ for both; Figure 9).

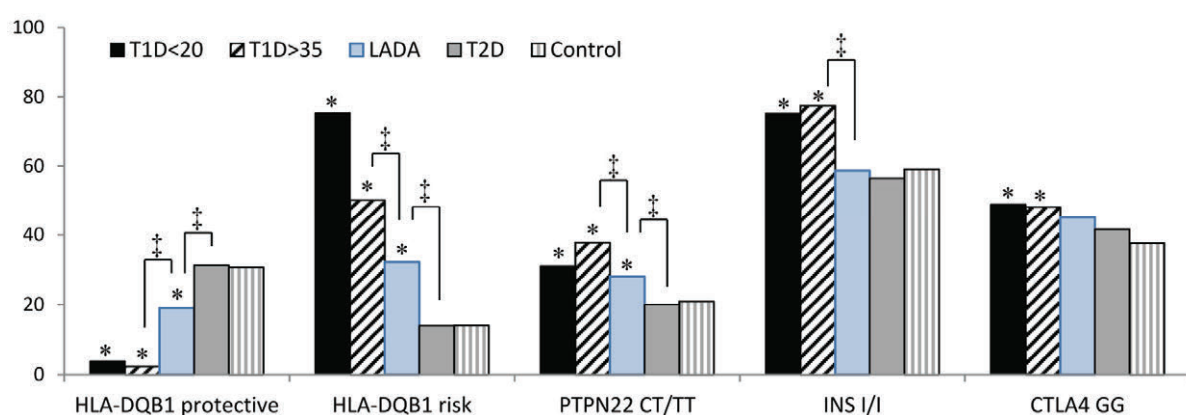


Figure 9 Genotype distribution of T1D-susceptibility variants in T1D_{<20} (n=158), T1D_{>35} (n=257), LADA (n=213), and T2D (n=648) patients, as well as non-diabetic control subjects (n=710). * Significant difference in genotype distribution compared with that of the control subjects: T1D_{<20}, $p\leq0.008$; T1D_{>35}, $p\leq0.003$; LADA, $p\leq0.03$. ‡ significant differences in genotype distribution between LADA and T1D_{>35} and/or T2D patients, $p\leq0.03$ (Study I). *HLA-DQB1*: risk, *02/*0302 or *0302/X; protective, *0602(3)/X or *0602(3)/*0301.

Among the T1D patients, association with age at diagnosis was observed for *HLA-DQB1*, whether analysed as genotype frequency in T1D_{<20} vs. T1D_{>35} (*02/*0302 or *0302/X, 75.3% vs. 50.2%, $p<0.000001$), or with age at diagnosis as a continuous variable in *HLA-DQB1* risk vs. neutral vs. protective genotype carriers (mean \pm SEM, 25.98 \pm 1.04 vs. 35.60 \pm 1.22 vs. 29.69 \pm 5.22 years, $p=0.004$). The frequencies of *PTPN22*-, *INS*-, and *CTLA4*-risk genotypes were similar in T1D_{<20} and T1D_{>35} patients (Figure 9). Two T2D-susceptibility gene variants, *TCF7L2* rs7903146 and *SLC30A8* R325W, were genotyped in the T1D_{>35} patients, but we observed no association for either of these variants (Figure 10).

In LADA patients, significant associations appeared for *HLA-DQB1**02/*0302 or *0302/X genotypes (control subjects vs. LADA patients: 14.1% vs. 32.2%, OR (95% CI): 2.90 (2.03-4.15), $p<0.0000001$) and the *PTPN22*-CT/TT genotypes (20.9% vs. 28.1%, OR: 1.48 (1.04-2.10), $p=0.03$). The frequency of the protective *HLA-DQB1**0602(3)/X or *0602(3)/*0301 genotypes was significantly lower in LADA patients than in non-diabetic control subjects (19.0% vs. 30.7%, OR: 0.53 (0.36-0.77), $p=0.001$). No association with LADA emerged for *INS* (I/I: 59.1% vs. 58.7%) or *CTLA4* (GG: 37.6% vs. 45.0%; Figure 9).

The T1D-susceptibility genotype frequencies in LADA patients differed significantly from the frequencies observed in T1D_{>35} with respect to *HLA-DQB1* ($p=0.00009$), *PTPN22* ($p=0.03$), and *INS* ($p=0.00002$), as well as the *HLA-DQB1*-protective genotypes ($p<0.000001$). Compared with T2D patients, the frequencies in LADA patients differed with respect to *HLA-DQB1*-risk ($p<0.000001$) and -protective genotypes ($p=0.0005$), as well as *PTPN22*-risk genotypes ($p=0.01$). The frequency of the *CTLA4* GG risk genotype in LADA patients did not differ significantly from its frequency in either T1D_{>35} or T2D patients (Figure 9).

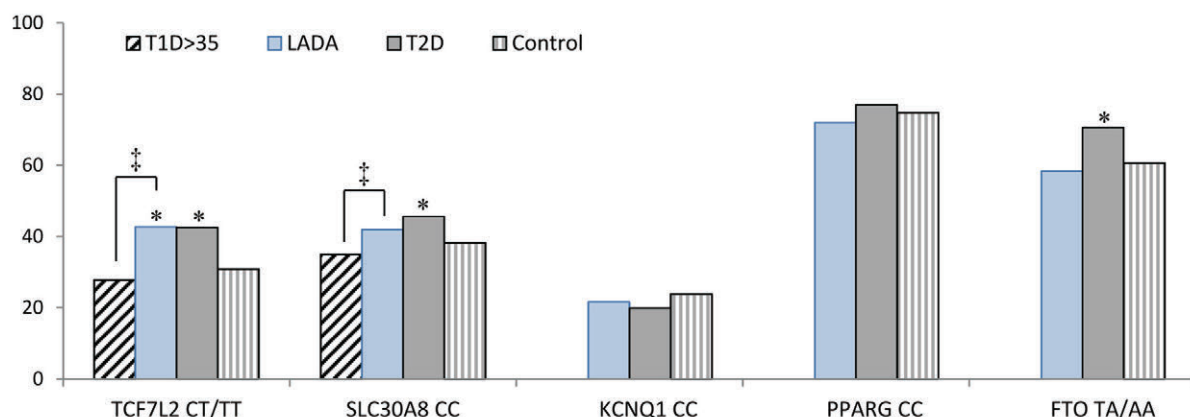


Figure 10 Genotype distribution of T2D-risk variants in T1D_{>35} (*TCF7L2*, $n=257$; *SLC30A8*, $n=274$), LADA (*SLC30A8*, $n=294$; all other, $n=213$), and T2D ($n=648$) patients, as well as non-diabetic control subjects (*SLC30A8*, $n=537$; all other, $n=710$). * Significant difference in genotype distribution compared with control subjects, *TCF7L2*: LADA, $p=0.001$; T2D, $p=0.0001$; *SLC30A8*: T2D, $p=0.026$; *FTO*: T2D, $p=0.003$. ‡ significant differences in genotype distribution between LADA and T1D_{>35} patients, *TCF7L2*: $p=0.0007$; *SLC30A8*: $p=0.049$ (Study III, unpublished data).

The association of T2D-susceptibility variants in *TCF7L2*, *SLC30A8*, *KCNQ1*, *PPARG*, and *FTO* was also assessed in LADA patients. Compared with non-diabetic control subjects, the frequency of the T2D-associated *TCF7L2* genotypes was significantly higher in LADA patients (CT/TT, 30.8% vs. 42.7%, OR: 1.67 (1.22-2.28), $p=0.001$), whereas no differences appeared for *SLC30A8* (CC, 38.2% vs. 43.2%), *KCNQ1* (CC, 23.8% vs. 21.6%), *PPARG* (CC, 74.7% vs. 71.9%), or *FTO* (TA/AA, 60.6% vs. 58.3%; Figure 10). Regarding *TCF7L2*, BMI was similar in LADA patients carrying the CT/TT and the CC genotypes (27.40 vs. 27.25 kg/m²), and the genotype frequencies were similar in non-overweight (BMI <25 kg/m², CT/TT: 44.9%) and overweight LADA patients (BMI \geq 25 kg/m², 38.2%; unpublished observation).

The frequencies of *TCF7L2*- and *SLC30A8*-risk genotypes in LADA patients differed significantly from frequencies observed in T1D_{>35} patients ($p=0.0007$ and $p=0.049$, respectively), whereas only the *FTO*-risk genotype frequency differed from the frequency in T2D patients (LADA vs. T2D, TA/AA: 58.3% vs. 70.6%, $p=0.006$ adjusted for BMI, Figure 10).

3. GENETIC HETEROGENEITY IN LADA (Studies I, III, and unpublished data)

Among LADA patients, GADA level was associated with clinical differences. Comparing clinical trait values across three groups, comprising patients with GADA levels in the lowest quartile (LADA_{low}; 32-43.9 IU/ml), patients with GADA levels in the two middle quartiles (LADA_{mid}; 44-278 IU/ml), and patients with GADA levels in the highest quartile (LADA_{high}; >278 IU/ml), insulin secretion (LADA_{low} vs. LADA_{mid} vs. LADA_{high}, mean \pm SD, fS-C-peptide: 0.56 \pm 0.35 vs. 0.54 \pm 0.47 vs. 0.31 \pm 0.33 nmol/l, $p=0.001$), lipid concentrations (HDL-cholesterol: 1.26 \pm 0.36 vs. 1.32 \pm 0.35 vs. 1.45 \pm 0.38 mmol/l, $p=0.03$; triglycerides: 1.74 \pm 1.02 vs. 1.60 \pm 0.81 vs. 1.51 \pm 1.64 mmol/l, $p=0.04$; LDL-cholesterol: 3.78 \pm 0.99 vs. 3.69 \pm 0.82 vs. 3.29 \pm 0.78 mmol/l, $p=0.005$), age at investigation (67.2 \pm 10.1 vs. 61.5 \pm 12.9 vs. 62.3 \pm 11.2 years, $p=0.02$), and BMI (28.3 \pm 4.4 vs. 27.4 \pm 5.0 vs. 25.8 \pm 4.0 kg/m², $p=0.01$) differed significantly. The LADA_{high} patients were more T1D-like, but differed significantly from the T1D_{>35} patients regarding fS-C-peptide levels ($p=0.001$). Conversely, the LADA_{low} patients were more T2D-like, but also between these two groups the fS-C-peptide levels differed significantly ($p=0.01$).

Among LADA patients, the frequency of the T1D-susceptibility variants in *HLA-DQB1*, *PTPN22*, *INS*, and *CTLA4* varied by GADA level and was highest in the LADA_{high} patients. The difference across the GADA quartiles was, however, only significant for *HLA-DQB1*-risk (*02/*0302 or *0302/X, LADA_{high} vs. LADA_{mid} vs. LADA_{low}, 44.2% vs. 32.4% vs. 19.6%, $p=0.03$) and -protective genotypes (*0602(3)/X or *0602(3)/*0301, 13.5% vs. 14.8% vs. 33.3%, $p=0.01$, Figure 11).

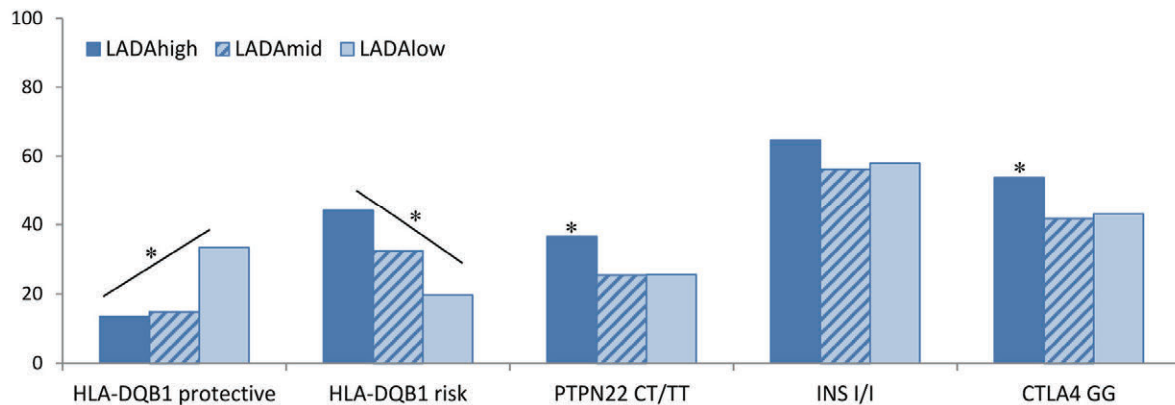


Figure 11 Genotype distribution of T1D-risk variants in LADA patients with GADA levels in the highest quartile (LADA_{high}, n=52), the two middle quartiles (LADA_{mid}, n=109), and the lowest quartile (LADA_{low}, n=52). *LADA_{high} vs. non-diabetic control subjects, *PTPN22*: p=0.009; *CTLA4*: p=0.02; across the GADA-level groups, *HLA-DQB1* risk: p=0.03; *HLA-DQB1* protective: p=0.01. *HLA-DQB1*, risk: *02/*0302 or *0302/X, protective: *0602(3)/X or *0602(3)/*0301 (Study I).

The *PTPN22*-risk genotype frequency did not differ between LADA_{mid}/LADA_{low} and the control subjects (25.3% vs. 20.9%, p=0.227); hence the association was restricted to the LADA_{high} group (p=0.009). Similarly, for *CTLA4*, an association emerged in the LADA_{high} subjects (control subjects vs. LADA_{high}, GG: 37.6% vs. 53.8%, p=0.02), even though no association occurred in comparison of control subjects and the entire LADA sample. For *INS*, not even the LADA_{high} group differed from the control group (Figures 9 and 11). The T1D-risk genotype frequencies observed in LADA_{high} were similar to frequencies in the T1D_{>35} patients; between the two groups, only the frequency of the *HLA-DQB1*-protective genotypes differed significantly (p=0.0003). Conversely, the genotype frequencies in LADA_{low} were similar to those observed in T2D patients (Figures 9 and 11).

The T2D-susceptibility gene variants in *TCF7L2*, *KCNQ1*, and *PPARG* made no contribution to heterogeneity in LADA, as the risk-genotype frequencies of these variants were independent of GADA level (Figure 12). However, compared with LADA_{mid}/LADA_{low}, the *SLC30A8* CC-risk genotype was significantly lower among LADA_{high} patients (45.6% vs. 32.0%, p=0.04). Compared with T2D patients, both *FTO* (p=0.009 adjusted for BMI) and *SLC30A8* (p=0.029) risk-genotype frequencies were significantly lower in LADA_{high} patients (unpublished data). And after splitting the LADA patients according to the GADA median, the *SLC30A8* CC genotype was significantly associated with LADA that had GADA levels below the median (LADA vs. control subjects, 47.4% vs. 38.2%, OR: 1.46 (1.0-2.1), p=0.049).

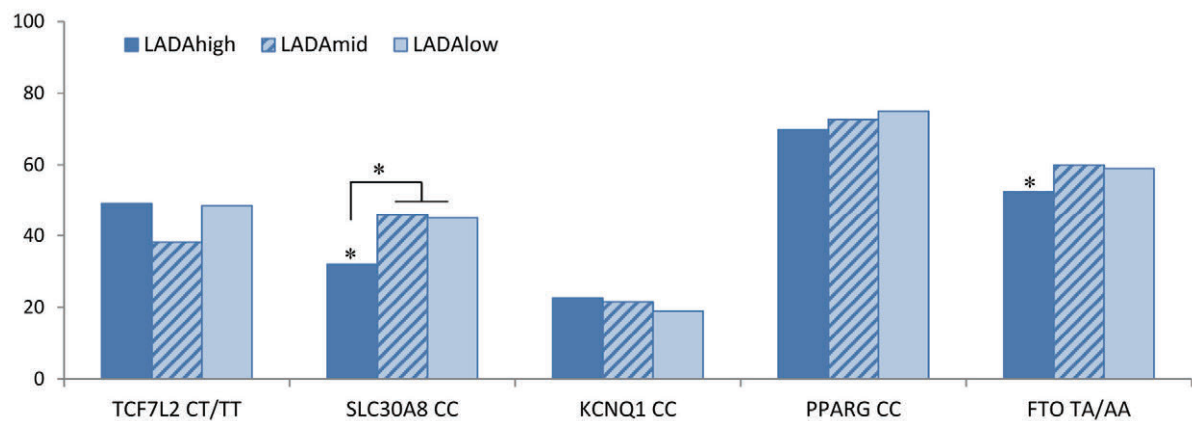


Figure 12 Genotype distribution of T2D-susceptibility variants in LADA patients with GADA levels in the highest quartile (LADA_{high}, n=52), the two middle quartiles (LADA_{mid}, n=109), or the lowest quartile (LADA_{low}, n=52). **SLC30A8*: LADA_{high} vs. LADA_{mid}/LADA_{low}, p=0.04 vs. T2D, p=0.029; *FTO*: LADA_{high} vs. T2D, p=0.009 (Study III and unpublished data).

Family history of T1D was also associated with genetic heterogeneity in LADA patients. Comparing LADA patients with a family history of both T1D and T2D with LADA patients having a family history of T2D only, the frequency of *HLA-DQB1**02/*0302 or *0302/X genotypes was higher (51.2% vs. 29.6%, p=0.001) and the frequency of *TCF7L2* CT/TT genotypes was lower (34.5% vs. 48.8%, p=0.05). Similar to *HLA-DQB1*, the *PTPN22* CT/TT genotypes were higher in patients with a family history of T1D and T2D than in patients with T2D only (23.9% vs. 33.7%); this difference was, however, non-significant. The frequency of *INS*-, *CTLA4*-, *SLC30A8*-, *KCNQ1*-, *PPARG*-, and *FTO*-risk genotypes was independent of family history (Unpublished data, not shown).

Similar to the observation in LADA patients, family history of T1D was also associated with a higher frequency of *HLA-DQB1*-risk genotypes among the T2D patients (*HLA-DQB1**02/*0302 or *0302, family history of T2D vs. family history of T1D and T2D: 12.9% vs. 23.0%, p=0.03). No differences in genotype frequencies were observable for *PTNP22*, *INS*, *CTLA4*, or any of the T2D-susceptibility variants regarding family history in T2D patients (Unpublished data, not shown).

4. PHENOTYPIC ASSOCIATIONS OF T1D- AND T2D-SUSCEPTIBILITY VARIANTS (Studies I, II, III, and unpublished data)

T1D-associated risk genotypes did not affect the clinical phenotype of the T1D patients, but were associated with the clinical phenotype of LADA and T2D patients. Among LADA patients, an increasing number of risk genotypes correlated with reduced insulin secretion (fS-C-peptide, 0 vs. 1 vs. ≥2 risk genotypes: 0.68 (0.65) vs. 0.44 (0.43) vs. 0.31 (0.60) nmol/l, p=0.015) and younger age at diagnosis (62.0 (17.5) vs. 54.5 (18.3) vs. 53.0 (18.5) years,

$p=0.04$). Among T2D patients, a similar trend appeared with respect to the fS-C-peptide (0.69 (0.43) vs. 0.68 (0.46) vs. 0.56 (0.51) nmol/l, $p=0.027$).

In the 2 764 initially non-diabetic subjects of the Botnia Prospective Study, T1D-associated risk variants in HLA-DQ, *PTPN22*, *INS*, and *CTLA4* were also associated with the clinical phenotype. At baseline, carrying a larger number of risk genotypes was associated with reduced insulin secretion (genetic risk score of ≤ 1 vs. 2 to 3 vs. ≥ 4 , insulinogenic index: 13.27 (16.27) vs. 12.69 (15.27) vs. 10.98 (13.06), $p=0.02$) and enhanced insulin sensitivity (insulin sensitivity index: 142 (111) vs. 144 (118) vs. 157 (127), $p=0.01$; Figure 13). During a mean follow-up of 8.1 ± 3.6 years, BMI increased and insulin sensitivity decreased independent of the risk genotypes, but subjects with a high genetic risk score showed reduced levels of insulin secretion at follow-up; hence, these subjects were less able to compensate for the increased demand for insulin (Figure 13).

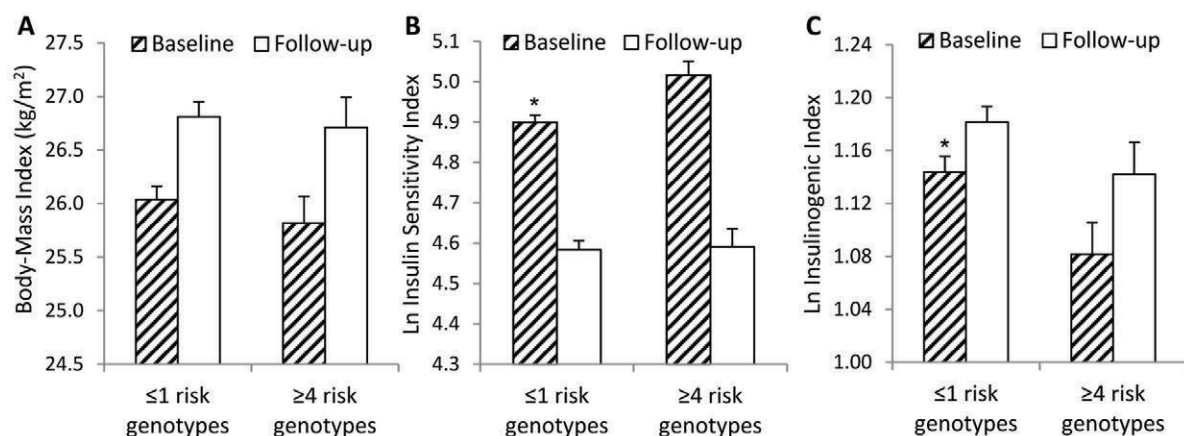


Figure 13 Effect of T1D-susceptibility variants on **A)** BMI, **B)** insulin sensitivity, and **C)** insulin secretion at baseline and follow-up. Data are mean values (SEM) logarithmically transformed for the insulin sensitivity index and the insulinogenic index. The group with a genetic risk score ≤ 1 ($n=1\ 116$) is compared with the group with a genetic risk score ≥ 4 ($n=297$) by a general linear model, and asterisks mark significant differences. Insulin sensitivity index at baseline, $p=0.004$; insulinogenic index at baseline, $p=0.017$ (Study II).

In these non-diabetic subjects, *HLA-DQB1*- and *PTPN22*-risk genotypes were, furthermore, associated with GADA (*HLA-DQ2.5/DQ8* or *DQ8*, OR: 1.7 (1.3-2.3), $p=0.0004$; *PTPN22* CT/TT, OR: 1.6 (1.2-2.2), $p=0.003$; Figure 14). Among the GADA-positive subjects, the *HLA-DQ2.5/DQ8* genotype was associated with a higher GADA level ($p=0.004$; data not shown). For *CTLA4*- and *INS*-risk genotypes, we observed no association with GADA (Figure 14). Compared with LADA patients, the frequency of the *HLA-DQB1*- and *PTPN22*-risk genotypes in the non-diabetic GADA-positive subjects was similar (*HLA-DQB1*: (*02/*0302 or *0302/X) 32.2% vs. (corresponding *DQ2.5/DQ8* or *DQ8*) 31.2%; *PTPN22* CT/TT: 28.1% vs. 28.0%; Figures 9 and 14).

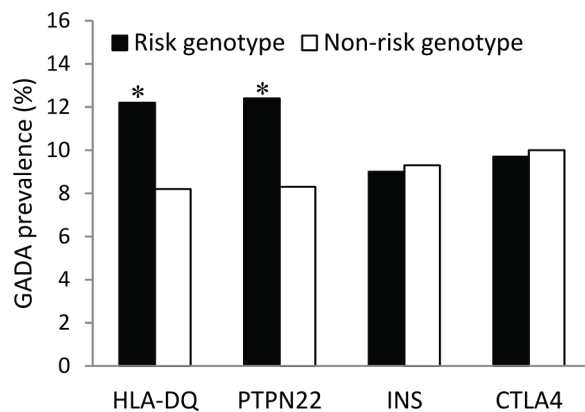


Figure 14 Frequency of GADA-positive subjects according to genotype. *HLA-DQ* risk: *DQ2.5/DQ8* or *DQ8*, $p=0.0004$; *PTPN22* risk: CT/TT, $p=0.003$; *INS* risk: I/I; *CTLA4* risk: GG. Differences were assessed with logistic regression (Study II).

Family history of T1D (FH_{T1}) was also associated with GADA in the non-diabetic subjects. With respect to *HLA-DQ* and *PTPN22*, the highest prevalence of GADA positivity appeared in subjects having both the risk genotype and FH_{T1} (Non-risk genotype and FH_{T1} - vs. risk genotype and $FH_{T1}+$; *HLA-DQ*, 7.7% vs. 16.3%, OR: 2.3 (1.5-3.6), $p=0.0002$; *PTPN22*, 7.8% vs. 20.5%, OR: 3.1 (1.9-5.0), $p<0.00001$; Table 7). The *INS*-risk genotype was only associated with GADA in combination with FH_{T1} (9.2% vs. 16.5%, OR: 1.9 (1.3-2.9), $p=0.001$), whereas no association between *CTLA4* and GADA was observable regardless of family history (Table 7).

Table 7 GADA prevalence by genotype and family history of T1D

Locus	Genotype/FH	GADA+ n (%)	OR (95% CI)	p-value
HLA	Non-risk/-	141 (7.7)	1.0	-
	Non-risk/+	31 (12.2)	1.7 (1.1-2.5)	0.016
	Risk/-	50 (10.8)	1.4 (1.0-2.0)	0.034
	Risk/+	28 (16.3)	2.3 (1.5-3.6)	0.0002
PTPN22	Non-risk/-	144 (7.8)	1.0	-
	Non-risk/+	36 (11.3)	1.5 (1.0-2.2)	0.036
	Risk/-	47 (10.4)	1.4 (1.0-1.9)	0.072
	Risk/+	23 (20.5)	3.1 (1.9-5.0)	<0.00001
INS	Non-risk/-	89 (9.2)	1.0	-
	Non-risk/+	17 (9.7)	1.1 (0.6-1.8)	0.845
	Risk/-	101 (7.6)	0.8 (0.6-1.1)	0.168
	Risk/+	41 (16.5)	1.9 (1.3-2.9)	0.001
CTLA4	Non-risk/-	126 (9.2)	1.0	-
	Non-risk/+	37 (14.5)	1.7 (1.1-2.5)	0.010
	Risk/-	65 (7.0)	0.8 (0.5-1.0)	0.071
	Risk/+	22 (12.9)	1.5 (0.9-2.4)	0.124

Differences between groups analysed with logistic regression; p-values (un-adjusted) compare the group with non-risk genotype and no family history of type 1 diabetes (FH_{T1}⁻) against the other groups for each gene variant (Study II).

In 2 764 initially non-diabetic subjects, a Cox regression analysis including known clinical T2D-risk factors (gender, age, BMI, FPG, GADA level, family history of T2D, and FH_{T1}) and the T1D-associated gene variants, showed no association for any of the T1D-risk variants (HLA-DQ2.5/DQ8, HLA-DQ8, HLA-DQ2.5, *PTPN22* CT/TT, *INS* AA, and *CTLA4* GG, HR: 1.0-1.2, p=0.455-0.977) with development of non-insulin-dependent diabetes. This analysis further showed that the previously reported association between FH_{T1} and development of non-insulin-dependent diabetes was independent of the T1D-associated variants (data not shown). In the cross-sectional study sample, as well, frequency of the T1D-risk variants and the *HLA-DQB1*-protective genotypes were similar in control subjects and in T2D patients (Figure 9).

Phenotypic associations were also assessed for the T2D-susceptibility R325W variant in *SLC30A8*. The T2D-associated *SLC30A8* genotypes were associated with reduced insulin secretion in both groups of non-diabetic subjects (incremental AUC S-C-peptide, CC/CT vs. TT: 1.88 (1.68) vs. 3.43 (1.88) nmol/l, p=0.049) and of LADA patients (2.34 (2.43) vs. 3.77 (3.76) nmol/l, p=0.036).

DISCUSSION

Genetic research was revolutionised by the advent of GWAS. In the last five years, this hypothesis-free approach has contributed a large number of novel susceptibility loci for both T1D and T2D, discoveries leading to new knowledge regarding the biological pathways underlying diabetes. Knowledge of the genetics underlying adult-onset T1D and LADA is, on the other hand, sparse. The genetics of T1D has thus far primarily been investigated in children <15 years; some studies have included a small group with T1D diagnosed >20 [20, 21, 148, 153] or >30 years [149, 150, 152, 154, 155], whereas research on large distinct groups of T1D patients >35 are lacking. With respect to the genetics of LADA, only a handful of studies have included 100 or more patients, with the observations from these LADA studies being extremely difficult to compare, as no consensus exists as to LADA diagnosis. The same problems apply to the clinical characterisation of LADA and of adult-onset T1D. Hence, based on earlier data, whether differences observed between LADA and T1D are solely attributable to differences in age at onset or reflections of true pathophysiologically differences is still an open question.

1. CLINICAL COMPARISON OF ADULT-ONSET T1D AND LADA

Our adult-onset T1D patients were comparable to young-onset T1D patients regarding metabolic traits and level of insulin secretion, which indicates that despite their marked differences in age at diagnosis, the pathogenesis underlying T1D patients diagnosed at <20 and >35 years is similar. These similarities were observed even though disease duration was significantly longer in the T1D patients diagnosed at <20 years. It is, however, possible that differences would have been observable at the time of diagnosis, as suggested by a report of lower insulin secretion in newly diagnosed T1D patients <20 years than in patients >20 years [20, 21].

Regarding metabolic traits and level of insulin secretion, LADA patients and T1D patients with age at diagnosis falling within a similar age-range clearly differed. This finding emphasises that differences observed in studies comparing LADA and T1D patients mainly diagnosed at <20 years [56, 70-72, 77] is more than an age-related phenomenon.

Also with respect to ZnT8A, patients with LADA and adult-onset T1D differed. Hence, ZnT8A were more prevalent among LADA (34%) than among adult-onset T1D patients (19%). The prevalence observed in T1D_{>35} patients was similar to the reported frequency of 24% in patients diagnosed at >30 years [26]. In our study, ZnT8A were not associated with age at diagnosis in T1D patients diagnosed at >35 years. But when looking at a larger age-at-onset spectrum, ZnT8A seem to be associated with younger age at onset, with a peak

prevalence in late adolescence of 80% [26, 242, 295, 296]. Overall, the prevalence observed in the LADA patients (34%) falls within the range of previous studies (4% to 42%) [296-300]. Variation among studies is probably due to small sample sizes (ranging from 47 to 193) and different criteria for LADA diagnosis, but is unlikely to be due to differences in GADA level, as we observed no correlation between ZnT8A and GADA level in the LADA patients. Furthermore, we observed no correlation between ZnT8A positivity and the phenotype of LADA patients; hence, despite the higher prevalence in LADA compared with that in adult-onset T1D patients, ZnT8A have limited value as a marker of progression to insulin therapy in LADA. This notion is in line with observations in the NIRAD Study [298]. However, ZnT8A positivity combined with an increased GADA level was associated with a more T1D-like phenotype in LADA patients with lower BMI and lower C-peptide levels.

Among T1D_{>35} patients, the ZnT8A prevalence was negatively correlated with disease duration, in line with observations in younger T1D patients [25]. The reduction in ZnT8A positivity may be a consequence of the progressive beta-cell loss resulting in lower amounts of ZnT8 antigens. On the contrary, we observed no correlation between ZnT8A prevalence and LADA duration. This lack of correlation may be a reflection of the lower degree of beta-cell destruction in these patients when compared with T1D patients or may indicate that ZnT8A are more persistent in LADA than in T1D_{>35} patients. This observation is, however, based on cross-sectional data, and needs to be replicated in a prospective study with several longitudinal ZnT8A measurements for each patient.

In relation to ZnT8A reactivity, the genotype of the R325W variant in *SLC30A8* encoding the ZnT8 protein is extremely interesting. This protein contains discrete epitopes depending on R325W genotype, giving rise to different ZnT8A subtypes, namely ZnT8A_R and ZnT8A_W (recognising the arginine and tryptophan epitope, respectively). Among the adult-onset T1D patients, the R325W variant was associated with ZnT8A antigen specificity. Hence, the arginine-coding CC genotype was associated with prevalence of the ZnT8A_R subtype, and the tryptophan-coding TT genotype was associated with the ZnT8A_W subtype in line with reports in T1D children [291]. The *SLC30A8* R325W genotype was not associated with the ZnT8A specificity among the LADA patients. This may indicate that the ZnT8A epitopes in T1D and LADA differ, and that the ZnT8A binding in LADA patients is less specific. In summary, the ZnT8A data indicate that LADA and adult-onset T1D patients differ immunologically, and our clinical data support the notion that LADA is a phenotype with a lower degree of beta-cell destruction than in T1D even when diagnosed at >35 years.

2. GENETIC COMPARISON OF ADULT-ONSET T1D AND LADA

The T1D-susceptibility variants in *HLA-DQB1*, *PTPN22*, *INS*, and *CTLA4* were all significantly associated with adult-onset T1D, as well as with T1D diagnosed at <20 years.

Genetic studies on T1D patients diagnosed at >35 years are few, but in children [136] and adults mainly diagnosed at <35 years, *HLA-DQB1* have consistently been associated with T1D [20, 21, 148-154]. With respect to *PTPN22*, *INS*, and *CTLA4*, association with T1D has been reported in children [136] and for *PTPN22* and *INS*, an association has also been evident in young adults aged between 15 and 40 years at diagnosis [154].

A study on twins indicates that compared with young-onset patients, the genetic component in T1D development in adult-onset patients is weaker [91]. This notion is supported by our results on *HLA-DQB1*, where the risk-genotype frequency was significantly higher among T1D patients diagnosed at <20 years than in patients diagnosed at >35 years, and by previous comparisons mainly of T1D patients diagnosed under and over the age of 20 years [20, 21, 148-154]. Variation in *HLA-DQB1*-risk genotype frequencies between populations and variation in age-at-onset limits for the groups included in each study, however, makes it difficult directly to compare the frequencies in our T1D patients >35 years with the frequencies in other reports. With respect to *PTPN22*, *INS*, and *CTLA4*, the frequencies of the risk genotypes were similar in T1D patients at >35 and at <20 years at diagnosis and, thus were not associated with age at onset of T1D. This conclusion is supported by observations in T1D patients diagnosed at between 15 and 40 years [154], whereas in T1D children, association with age at diagnosis has been apparent for *INS* [166] and for *PTPN22* according to one [165] but not all studies [161, 166]. Furthermore, the protective *HLA-DQB1**0602(3)/X or *0602(3)/*0301 genotypes were equally rare in T1D patients >35 (2.3%) and <20 years (3.8%). Taken together, our data thus suggest that besides *HLA-DQB1**02/*0302 and *0302, the genetic predisposition toward T1D in children and adults is similar. Differing ages at onset in T1D might instead be due to differing overall genetic load or different accumulation of environmental exposures. It is, however, possible that the genetic basis for a delayed age at onset is represented by gene variants exclusively associated with adult-onset T1D. This possibility is thus far barely explored.

*HLA-DQB1**02/*0302 or *0302 and *PTPN22*-CT/TT genotypes were significantly associated with LADA in general, and *CTLA4* CT60 GG with LADA with high GADA levels. With respect to *HLA-DQB1*, this is in line with others' reports [56, 65, 280, 285], and with respect to *PTPN22* the association is in agreement with the Skåne Diabetes Register Study [280] but in disagreement with the HUNT Study [65]. This disagreement is likely due to the smaller sample size in the HUNT Study, where *PTPN22*-risk genotype frequencies both in LADA patients and in control subjects were similar to the frequencies observed in both our study and the Skåne Diabetes Register Study.

We observed no association between the *INS* -23HphI variant and LADA. However, the role of this variant in LADA has been disputed. The UKPDS (*INS* risk genotype frequency: LADA, 69%; control subjects, 49%) and the Skåne Diabetes Register Study (LADA, 69%;

control subjects, 55%) have reported an association between the variant and LADA [280, 284], whereas the Botnia Study (LADA, 47%; control subjects, 55%; [56]) and the HUNT Study (LADA, 56%; control subjects, 54%; [65]) report no association. This discrepancy may be due to differing patient recruitment. In the UKPDS Study, the LADA inclusion criterion for age-at-diagnosis was ≥ 25 years and for insulin-free time after diagnosis only 3 months, which might lead to inclusion of adult-onset T1D patients among the LADA patients. A similar classification problem might exist in the Skåne Diabetes Register Study, where no insulin-free period was required for the LADA patients. The relatively high frequency of *INS*-risk genotypes among the LADA patients in the Skåne Diabetes Register Study may be an indication of T1D contamination in the LADA sample. This type of contamination would clearly affect the results, as we show that the frequency of the *INS*-risk genotype among young- and adult-onset T1D patients was similar. It is also possible that this discrepancy is due to different proportions of LADA patients with high GADA levels or a family history of T1D in each study, as the *INS*-risk genotype was somewhat increased in both the LADA patients with high GADA levels and those LADA patients with a family history of T1D.

Compared with adult-onset T1D patients (>35 years), frequencies of the HLA-DQB1*02/*0302 and *0302, the *PTPN22* R620W CT/TT, and the *INS* -23HphI I/I genotypes in LADA patients were significantly lower, and frequency of the *HLA-DQB1*-protective genotypes was significantly higher. In studies comparing LADA patients and T1D patients diagnosed primarily <35 years, a similar difference emerged with respect to the *HLA-DQB1*-protective genotypes [56, 65, 280]. However, there is a discrepancy with respect to the HLA-risk genotypes. Hence, the HUNT Study [65] and the Botnia Study [56] agree with our observation, but in the Skåne Diabetes Register Study the HLA-risk genotype frequency in LADA patients was similar to its frequency in T1D patients diagnosed at <35 years [280]. This discrepancy may be ascribed to T1D contamination in the LADA sample, as already mentioned, or to a higher proportion of LADA patients with high GADA levels in the Skåne Diabetes Register Study. The latter explanation is supported by the correlation between GADA level and *HLA-DQB1*-risk genotype frequency observed in our present study and by others [56, 62, 65]. The HLA-DQB1*0602(3)/X or *0602(3)/*0301 protective genotypes also differed significantly by GADA level among LADA patients. Thus, the hypothesis of a higher frequency of LADA with a high GADA level in the Skåne Diabetes Register Study is further supported by the lower frequency of *HLA-DQB1*-protective genotypes in the Swedish LADA patients (8.1%), as compared with our LADA patients (19%). Our results clearly support a strong correlation between GADA level and prevalence of T1D-associated risk genotypes, as also the *PTPN22*- and *CTLA4*-risk genotype frequencies were higher in the LADA patients with high GADA levels. For *PTPN22*, this notion is supported by the NIRAD Study, where

association between the locus and LADA was only observed in patients with high GADA levels [282].

Studies on T2D-susceptibility variants in LADA are few. In our sample, only the CT/TT genotype of the *TCF7L2* rs7903146 variant was associated with LADA in general. The *TCF7L2* association with LADA was also reported in the Skåne Diabetes Register Study [280], and in a meta-analysis comprising five studies [281]. Despite reporting risk-genotype frequencies similar to those in the Skåne Diabetes Register Study, the HUNT Study observed no association [65]. Taken all together, these data support an association between the *TCF7L2* variant and LADA; the lack of association in the HUNT Study is most likely due to its smaller sample size.

Based on the correlation between GADA level in the LADA patients and distribution of the T1D-susceptibility genotypes, we expected an inverse correlation between GADA level and the T2D-susceptibility genotypes. This correlation was observed for *SLC30A8* R325W and *FTO* rs9939609, and the *SLC30A8* variant was associated with LADA with low GADA levels. Supporting our results, the HUNT Study reported a similar correlation for another *FTO* variant (rs8050136), and a non-significant trend for the *SLC30A8* R325W variant [65]. The distribution of the *TCF7L2* rs7903146, *KCNQ1* rs2737895, and *PPARG* P12A genotypes was independent of GADA level. For *TCF7L2*, this is contrary to the NIRAD Study reporting an association between the locus and LADA only in patients with low GADA levels [283]. It has also been suggested that the association between the *TCF7L2* variant and LADA is stronger among non-overweight LADA patients [281]; this observation was not, however, supported by our data.

Compared with adult-onset T1D, the frequencies of the T2D-associated *TCF7L2* and *SLC30A8* genotypes in LADA patients were significantly higher. Compared with T2D patients, the frequency of the *FTO*-risk genotype in LADA patients was lower, even when adjusted for BMI. In sum, with respect to the T2D-susceptibility gene variants, LADA differs from both adult-onset T1D and from T2D. This conclusion is further supported by data on the T1D-susceptibility gene variants; LADA differed from both adult-onset T1D and from T2D, with respect to *HLA-DQB1*-risk and -protective genotypes, as well as *PTPN22*-risk genotypes, and from adult-onset T1D also with respect to the *INS*-risk genotype. These genetic differences between LADA and adult-onset T1D may be a contributing factor to the milder reduction in beta-cell function observed in LADA. And the genetic differences combined with the observed clinical and immunological differences between these two diabetic subgroups imply that the pathophysiological process underlying LADA differs from the process underlying T1D. Hence, our data do not support the categorisation of LADA as an age-related extension of childhood T1D, but instead suggest that LADA is a slowly progressive form of autoimmune diabetes distinct from T1D. The distinction between LADA

and T1D is supported by studies showing differences between the two diabetic subgroups regarding GADA-epitope recognition and -IgG subclass profile [71, 301-305] as well as regarding cellular immune reactivity to islet proteins [306]. Furthermore, in sera from T1D patients, 60% of ICA staining is blocked by GADA and IA-2A, whereas only 37% is blocked in sera from LADA patients. These results indicate that yet-unidentified autoantibodies may be more prevalent in LADA than in T1D [307].

3. GENETIC HETEROGENEITY IN LADA

Clinically, those LADA patients with low GADA levels were more T2D-like, whereas those with high GADA levels were more T1D-like. GADA level was also associated with genetic differences with respect to *HLA-DQB1*-risk and -protective genotypes as well as risk genotypes in *PTPN22*, *CTLA4*, *SLC30A8*, and *FTO*. Thus, our results clearly support genetic and phenotypic heterogeneity within LADA attributable to the strength of GADA reactivity, which possibly signifies the magnitude of autoimmune destruction of pancreatic beta cells. The phenotypic difference between patients with high and low GADA levels may be clinically relevant and represent a central distinguishing factor for choosing insulin therapy or lifestyle-changing intervention. In the NIRAD Study, GADA levels show a bimodal distribution, facilitating an easy subdivision into two separate LADA subtypes [62]. In other LADA cohorts, including ours, the distribution of GADA levels is continuous, making it challenging to determine the cut-off between high and low GADA levels.

Even a low GADA level may represent an autoimmune-disease process, where the beta-cell function is relatively well-preserved. This notion is supported by the observation of significantly lower insulin secretion even in LADA patients with low GADA reactivity compared with T2D patients. Genetically, the LADA patients with a low GADA level and the T2D patients were similar. Thus, low-level GADA positivity could also result from false-positive GADA reactivity in the assay, in which case these subjects would be T2D patients incorrectly diagnosed with LADA. It is impossible to eliminate this potential misclassification of T2D patients, as no alternative diagnostic test that shows higher specificity exists.

Compared with adult-onset T1D patients, even LADA patients with a high GADA level had significantly higher levels of C-peptide and had a significantly higher frequency of both the *TCF7L2*-risk genotypes and of the *HLA-DQB1*-protective genotypes. These observations suggest that even though LADA patients with high GADA levels are more T1D-like, they are still phenotypically and genetically distinct from adult-onset T1D patients.

Overall, the autoimmune attack seems to be milder, and the disease progression seems to be slower in LADA patients than in adult-onset T1D patients, especially in LADA patients with low GADA concentrations. These differences may be a reflection of LADA patients' lower genetic predisposition. Family history of T1D was also associated with genetic

heterogeneity in LADA, which suggests that family history is a factor that should be considered in study of LADA genetics.

4. PHENOTYPIC ASSOCIATIONS OF T1D- AND T2D-SUSCEPTIBILITY VARIANTS

Although we have reported an association between family history of T1D and development of non-insulin dependent diabetes [54], T1D-susceptibility variants in *HLA-DQB1*, *PTPN22*, *INS*, and *CTLA4* were not associated with non-insulin-dependent diabetes in the same study sample. Thus, these T1D-susceptibility variants do not seem to contribute to a direct genetic overlap between T1D and T2D. Well-powered studies of identified T1D-susceptibility variants in T2D and *vice versa* also give little support to genetic overlap between the two diabetic subtypes. Genetic overlap cannot, however, be excluded. Thus far, only common T1D- and T2D-susceptibility variants have been identified, representing only a fraction of the genetic heritability. It is therefore possible that yet-unidentified rare variants will contribute to a genetic overlap. As for the majority of the T1D- and T2D-susceptibility loci identified, causal variants underlying the statistical associations remain unidentified. The causal variants might have higher penetrance than do the proxies identified in the GWAS. Assessing the true causal variants would therefore increase the statistical power considerably. One speculation is that it is the variants associated with insulin resistance that are shared between T1D and T2D [308], as exemplified by *PPARG* [248, 270-272]. To date, only a few of the T2D-susceptibility variants identified affect insulin resistance. These variants seem to have lower effect sizes than do variants associated with beta-cell function and are therefore harder to identify and require larger sample sizes for study. Furthermore, because the T2D-susceptibility variants have thus far mainly been analysed in T1D children, it is possible that T2D-associated variants are associated with adult-onset T1D. This hypothesis is, however, not supported by the observance of a lack of association between adult-onset T1D and the T2D-susceptibility variants in *TCF7L2* (rs7903146) and *SLC30A8* (R325W).

The association between an increased number of T1D-susceptibility variants and reduced C-peptide levels in T2D patients, however, points to a possible involvement of the T1D-susceptibility variants in the pathogenesis of T2D. The *HLA-DQB1*, *PTPN22*, *INS*, and *CTLA4* T1D-susceptibility variants - combined in a genetic risk score - were also associated with reduced C-peptide levels in both non-diabetic subjects and LADA patients, which indicates that these variants signify a reduced insulin-secretory capacity which may lead to development of diabetes in susceptible individuals. In non-diabetic subjects, the *HLA-DQ2.5/DQ8* and *PTPN22* CT/TT genotypes were associated with the presence of GADA. Both associations were accentuated in subjects with a family history of T1D, and in this subgroup, association with GADA was also evident for the *INS*-risk variant. For HLA, our

results support the hypothesis that suggests that autoantigenic targets change over time [23, 30, 187-190, 195]. Both in T1D patients [196, 309] and in non-diabetic subjects, GADA expression seems to be independent of the CT60 variant in *CTLA4*.

Frequencies of the *HLA-DQB1* and *PTPN22*-risk genotypes in LADA patients and among GADA-positive non-diabetic adults were similar. This similarity indicates that these T1D-susceptibility variants are associated with initiation of the autoimmune process leading to generation of autoantibodies, but additional factors are required to cause overt diabetes in adults. In children, the *PTPN22* variant has been associated with development of persistent autoantibodies [310], but also, after the appearance of autoantibodies, with progression to T1D [311].

Identification of the T2D-associated *SLC30A8* R325W variant highlighted the important role of the ZnT8 zinc transporter in insulin secretion [203, 204, 237, 238]. And functional studies have shown that this transporter is a potential new drug target in T2D, as increased transporter function could potentially enhance insulin secretion [240]. Our results indicate that this type of drug may be effective in LADA patients as well, as the CC/CT genotypes of the *SLC30A8* R325W variant were associated with reduced insulin secretion in these patients and in non-diabetic control subjects. A ZnT8-targeted drug may be especially relevant in LADA patients with low GADA levels, because the residual beta-cell mass in these patients seems to be greater than in adult-onset T1D patients and in LADA patients with high GADA levels.

CONCLUSIONS AND PERSPECTIVES

- LADA shared a genetic predisposition with both T1D (*HLA-DQB1*, *PTPN22*, *CTLA4*) and T2D (*TCF7L2*, *SLC30A8*), and differed from adult-onset T1D clinically, immunologically, and genetically.
- Genetic heterogeneity in LADA was linked to GADA reactivity (*HLA-DQB1*, *PTPN22*, *CTLA4*, and *SLC30A8*) and family history of T1D (*HLA-DQB1* and *TCF7L2*).
- *HLA-DQ2.5/DQ8* and *PTPN22* CT/TT genotypes were associated with GADA in non-diabetic adults and carrying of an increased number of T1D-susceptibility genotypes was associated with reduced insulin secretion, evident in both LADA and T2D patients as well as in non-diabetic adults.

LADA is genetically heterogeneous, and it appears to be a slowly progressive form of autoimmune diabetes distinct from T1D. T1D-susceptibility variants in *HLA-DQB1*, *PTPN22*, *INS*, and *CTLA4* were not directly associated with development of non-insulin dependent diabetes, but signified a mild degree of beta-cell destruction in non-diabetic adults (reduced insulin secretion and GADA) as well as in LADA and T2D patients (reduced insulin secretion).

Our data have added to the genetic knowledge of LADA, but the question of whether unique LADA-susceptibility loci exist remains unanswered. One obvious way to answer this question is to conduct a GWAS of LADA. This hypothesis-free approach could potentially reveal loci shared between LADA and T1D or T2D, and reveal novel loci exclusive to LADA. Such novel loci may point to molecular pathways distinguishing the LADA phenotype.

A major obstacle to performing a well-powered GWAS study is, however, the requirement for a substantial sample of well-characterized LADA patients. One way to overcome this would be to combine existing LADA cohorts through international collaboration. This strategy would, however, require a consensus as to the unit for GADA measurement, as to the threshold for GADA positivity, and as to general diagnostic criteria for LADA. The WHO units/ml is defined in relation to a standard reference [308] and has been validated in international workshops [309]. This would therefore be the natural choice for a consensus GADA unit. Achieving a consensus on diagnostic criteria is more challenging, because choice of a cut-off limit for GADA reactivity, for age at onset, and for insulin-free period after diagnosis always will be arbitrary, and these cut-off limits may fail to

represent distinct clinical differences. It is almost inevitable that the characteristics of LADA patients will vary between studies; it will therefore also be important to seek ways to account for this when performing genetic analyses. Adult-onset T1D patients would also be of great interest to explore in a genome-wide setting to uncover the genetic cause of their slower autoimmune beta-cell destruction. Associated genetic loci may point to biological pathways underlying their delayed disease onset and hence reveal important therapeutic targets for T1D. Moreover, further exploration of the clinical consequences of GADA positivity and level in adults prior to and following development of diabetes would be of great importance to determine whether autoantibody status truly affects the disease course.

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